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DIMINISHED KETOGENESIS IN LIVER INJURY. I

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INTRODUCTION

THE liver is the site of the major biosynthetic and oxidative processes in the body. It is not surprising, therefore, that disease of this organ produces such profound metabolic alterations. Abnormalities of carbohydrate and protein metabolism have been demonstrated in animals and patients with liver injury.[1, 2] It might be anticipated, then, that disturbances of fat metabolism would also exist.

It is the purpose of this paper to present studies of certain aspects of fat metabolism in animals and human subjects with liver injury. As an approach to an evaluation of fat metabolism, the formation of ketone bodies under conditions leading to endogenous fatty acid catabolism as well as in response to exogenous fatty acid administration has been investigated.

MATERIALS AND METHODS

A. *Animal Experiments.*—In these experiments liver injury was induced by the administration of an ethionine-containing diet. This material, an ethyl analogue of methionine, has been shown by Koch-Weser[3] to produce lesions of hepatic necrosis and cirrhosis in rats. The mechanism by which the injury is produced is not fully understood, though an effect upon protein synthesis is suspected. Female rats of the Wistar strain, weighing from 110 to 150 grams, were placed on a basal diet to which dl-ethionine was added to a final

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concentration of 0.5 per cent. The basal diet consisted of sucrose 75 per cent, vitamin-free casein* 16 per cent, salt mixture (HMW) 4 per cent, and corn oil 5 per cent. The following vitamins were added to each kilogram of the diet: thiamine—10 mg., pyridoxine—10 mg., niacinamide—40 mg., riboflavin—20 mg., calcium pantothenate—40 mg., choline chloride—300 mg., menadione—5 mg., vitamin A—60,000 units, and irradiated ergosterol—8,500 units. Control rats were maintained on the basal diet without ethionine and were pair-fed in amounts equal to those consumed by the ethionine-fed animals. All studies were carried out after 21 to 35 days on the diet, at which time well-defined histologic evidence of hepatic changes was demonstrable.

Ketone formation was studied in both intact rats and in the isolated liver slice. The method of Greenberg and Lester[4] was used in these determinations.

B. *Human Experiments.*—Twenty-six medical students, 16 hospitalized patients without liver disease, and 27 patients with varying forms and stages of hepatic cirrhosis were studied. These patients were on the wards of Barnes Hospital, St. Louis City Hospital, and Cochran Veterans Hospital. Patients were fed usual hospital diets during the study, the cirrhotic group on the whole receiving a lower salt intake. Some of the hospitalized controls were cardiacs also on low salt diets. In these patients blood ketone levels were measured in response to a standard 18-hour period of fasting. The effect of glucose on blood ketones, pyruvic, and lactic acids was also studied. Pyruvate was measured by the method of Friedemann and Haugen,[5] lactate by the Barker and Summerson procedure,[6] and glucose by the Nelson method.[7]

RESULTS

A. *Animal Experiments.*—To test the ability of the rat with liver damage to metabolize fat, Na butyrate (12.5 per cent in aqueous solution in a dosage of

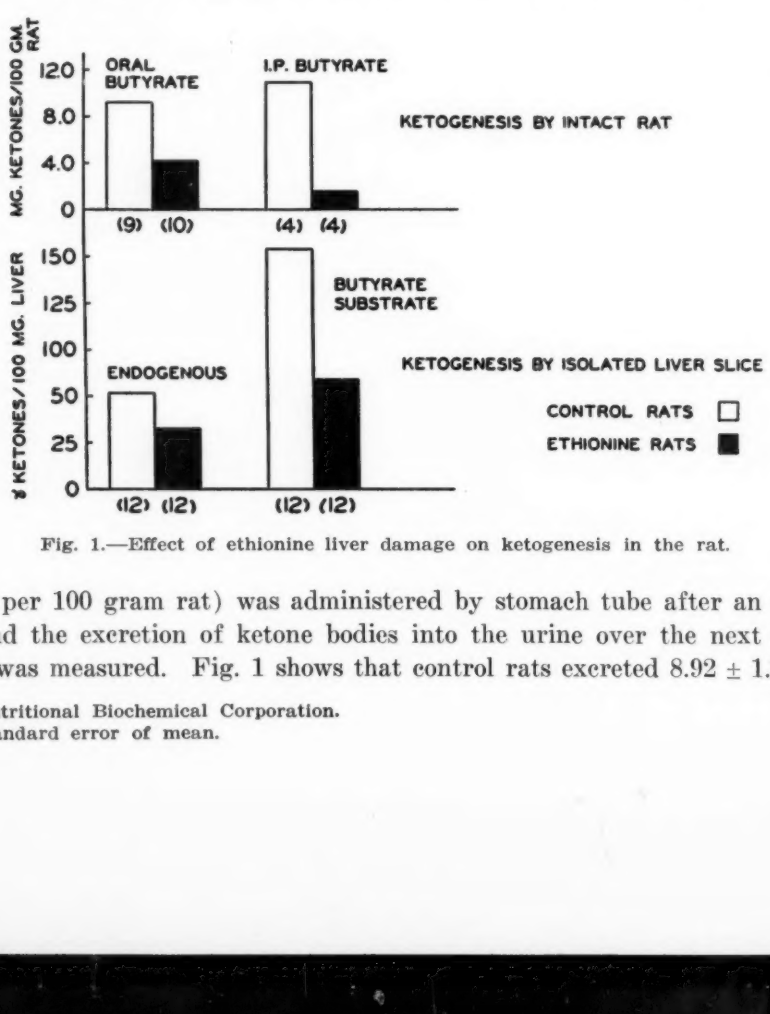

Fig. 1.—Effect of ethionine liver damage on ketogenesis in the rat.

0.5 ml. per 100 gram rat) was administered by stomach tube after an 18-hour fast, and the excretion of ketone bodies into the urine over the next 24-hour period was measured. Fig. 1 shows that control rats excreted 8.92 ± 1.39† mg.

*Nutritional Biochemical Corporation.
†Standard error of mean.

of ketones per 100 grams of rat, while the ethionine animals excreted only 4.14 ± 1.3 mg. Under the conditions of oral fat feeding, a significantly ($t = 2.9$) smaller quantity of ketones are excreted by rats with liver damage.

Since ethionine has been shown to produce pancreatic lesions,[3] the possibility was considered that butyrate was poorly absorbed in the ethionine rats. To rule out this possibility, butyrate was administered intraperitoneally. Reference to Fig. 1 shows that ethionine-fed animals manifested a ketonuria which was less than 25 per cent of the controls after parenteral fatty acid administration.

The next group of experiments was performed with isolated liver slices obtained from control and ethionine-fed rats. In this way a more direct measure of hepatic ketogenesis could be obtained since extrahepatic oxidation and renal excretion of ketones were eliminated. Liver slices obtained from 18-hour fasted rats were studied for (1) endogenous ketone formation and (2) ketone formation from a butyrate substrate. Approximately 200 mg. of tissue was incubated in Warburg vessels in 3 ml. of Ringer-phosphate buffer at pH 7.4. The atmosphere was O_2, temperature 37° C., and the incubation period 2 hours. At the end of incubation, proteins were precipitated by barium-zinc reagents and ketones were determined and recorded as micrograms per 100 mg. of tissue per 2 hours. In experiments with substrate present, 15 μM of Na butyrate per vessel was added. Fig. 1 shows the results obtained. Endogenous ketone formation in control livers was 69.0 ± 3.2, while ethionine livers formed only 29.8 ± 1.1 mcg. In the presence of a fatty acid substrate, the difference between the two groups was even more striking. Control liver slices formed 156 ± 8.5 mcg. of ketones, while the experimental group formed 52.5 ± 2.7 mcg.

It was concluded from these observations that in the experimental animal with liver damage the hepatic formation of ketone bodies from fatty acids is impaired.

B. *Human Experiments.*—In view of the above it seemed of interest to investigate ketone metabolism in the human subject with liver disease. Since fasting is known to induce an accelerated catabolism of fatty acids and associated ketosis, human subjects were fasted for an 18-hour period and blood ketone levels were measured. Fig. 2 shows the results obtained. The ketone level in subjects without liver disease was 2.28 ± 0.47 mg. per cent for nonhospitalized and 2.06 ± 0.59 mg. per cent for hospitalized controls. The cirrhotic group had a level of 0.94 ± 0.22 mg. per cent. It is apparent that cirrhotic subjects show a significantly diminished ketogenesis under these conditions. In order to determine whether a correlation exists between severity of hepatic disease and ketogenesis, a rough classification of the severity of liver disease on the basis of clinical and chemical information was made and compared with fasting ketone levels. Reference to Table I shows that, on the whole, those subjects with the lowest fasting ketone levels were most severely ill. However, some overlap does exist, indicating that the correlation is not perfect. This is most striking in the first case in the 4+ group. The patient died soon after the studies were completed. The liver at autopsy was described as a far-advanced portal cirrhosis. It is possible that delayed renal excretion of ketones in these preterminal patients explains the elevated blood ketone levels observed.

Since carbohydrate metabolism is known to be intimately related to both fatty acid synthesis and degradation, an effort to study the relationship between carbohydrate and ketone metabolism in these subjects was made. Seventeen controls and fifteen cirrhotic subjects were given a standard oral glucose tolerance test after the development of fasting ketonemia. Pyruvate, lactate, glucose,

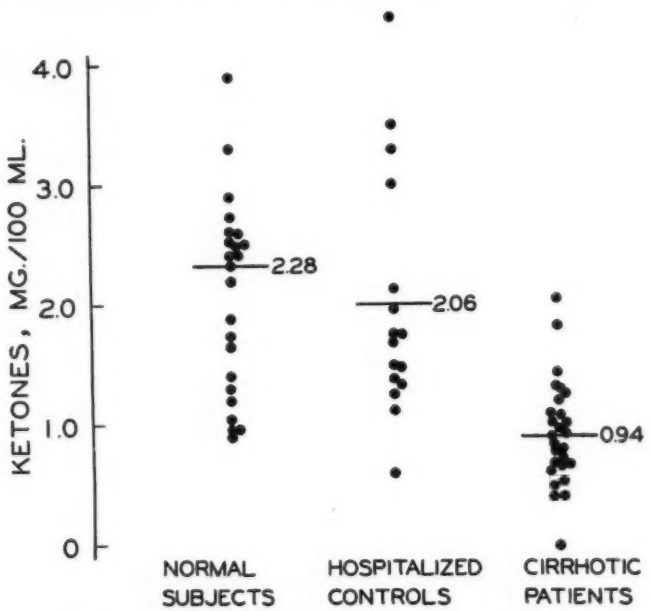

Fig. 2.—Effect of 18-hour fast on blood ketone level.

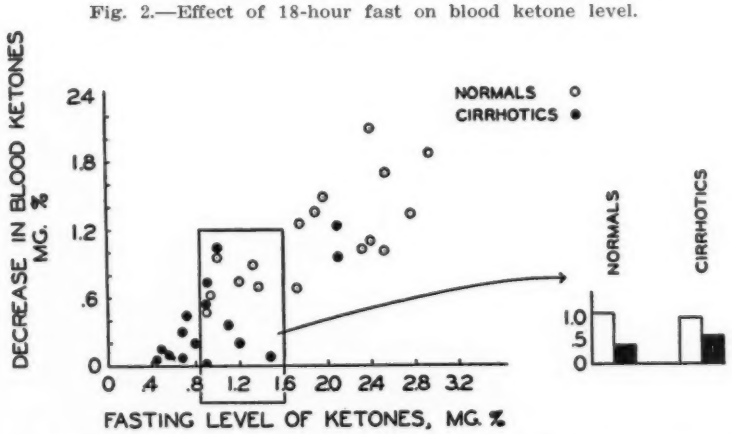

Fig. 3.—Relation between initial ketone level and magnitude of the glucose effect.

and ketone blood levels were measured at intervals of one and two hours following ingestion of glucose. No consistent differences were observed with regard to levels of pyruvate, lactate, and glucose. However, a striking decrease was observed in the ketone levels in the controls, while only a small change was observed in the cirrhotic group. Control ketone levels decreased by 1.15 ± 0.12 mg. per cent, while the cirrhotic levels decreased by 0.35 ± 0.08 ($t = 5.4$).

TABLE I. RELATION BETWEEN FASTING KETONEMIA AND SEVERITY OF LIVER DISEASE

SEVERITY OF LIVER DISEASE	KETONES MG./100 ML. MEAN LEVEL		DIAGNOSIS
		2.06	Alcoholic cirrhosis
		0.97	Alc. cirrhosis
		0.89	Alc. cirrhosis
		0.83	Alc. cirrhosis
		0.80	Alc. cirrhosis
		0.79	Fatty liver and alc. cirrhosis
		0.75	Fatty liver and alc. cirrhosis
4+	0.73	0.70	Hemachromatosis
		0.69	Hemachromatosis
		0.63	Cirrhosis and cancer
		0.55	Alc. cirrhosis
		0.50	Hemachromatosis
		0.44	Alc. cirrhosis
		0.41	Alc. cirrhosis
		0.00	Alc. cirrhosis
		1.10	Alc. cirrhosis and cancer
		1.05	Fatty liver
3+	1.14	1.03	Biliary cirrhosis
		1.38	Alc. cirrhosis
		1.09	Alc. cirrhosis and shunt
2+	1.05	0.73	Alc. cirrhosis
		1.32	Alc. cirrhosis
		1.82	Fatty liver, P.A.
		1.43	Alc. cirrhosis, cortisone
1+	1.44	1.32	Biliary cirrhosis
		1.20	Alc. cirrhosis

Since the initial fasting ketone level of the cirrhotic patients was lower than the controls, it seemed possible that the smaller drop in ketones following glucose was related to this low initial level. In Fig. 3 the initial ketone level is plotted against the change in ketone level obtained after glucose. It may be seen that despite initially similar levels in 7 cirrhotics and 6 controls, the fall in ketones was greater in the control group ($t = 17.0$). This observation appears to indicate that not only do patients with liver disease manifest diminished ketogenesis on starvation, but they also fail to respond to the influence of glucose ingestion on ketone levels despite apparently normal formation of pyruvate and lactate from glucose.

DISCUSSION

Essentially, two basic observations have been made. These are (1) that animals with liver injury and many patients with cirrhosis of the liver have diminished ketone formation under the stress of starvation plus a high intake of fat, or with starvation alone, and (2) that certain of these cirrhotic patients, despite grossly normal carbohydrate metabolism, have defective "integration" between the carbohydrate and fat pathways.

These findings suggest that fatty acid conversion to acetoacetate is interfered with in certain forms of liver injury. In addition, the partial failure of the cirrhotic ketone level to respond to glucose, despite the formation of adequate amounts of lactate and pyruvate, points to some defect in pyruvate activation.

A common enzymatic mechanism, coenzyme A activation, is involved in all these processes, namely, fatty acid breakdown, acetoacetate formation, and pyruvate utilization.

It would seem likely, then, that either a deficiency in CoA or in its activation occurs in liver injury and is responsible for the defective ketogenesis observed.

In terms of the over-all metabolic picture of the individual with liver disease, defective fatty acid metabolism may be of serious consequence. It is suggested that during periods of nutritional stress (starvation), the normal protein-sparing mechanism of increased fatty acid catabolism is inadequate and may contribute to the hypoproteinemia of liver disease.

SUMMARY

Diminished ketogenesis has been observed in rats with ethionine-induced liver injury. This defect is apparent in both the intact animal and in the isolated liver slice. Human subjects with cirrhosis of the liver appear to form considerably less ketones in response to starvation than do normal controls. In addition, the ketonemia of the human cirrhotic appears to respond differently to the administration of glucose as contrasted with the normal.

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A MECHANISM FOR DIMINISHED KETOGENESIS IN EXPERIMENTAL LIVER INJURY. II

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IMPAIRMENT of ketogenesis during fasting has been described in human subjects with severe hepatic dysfunction, as well as in the intact animal and the isolated liver slice of rats with experimentally induced liver injury.[11] Defective pyruvic acid metabolism in human subjects[2, 4, 14] and in rats with liver injury has also been reported.[5, 10, 13]

In an effort to define more precisely the nature of these defects in pyruvic and fatty acid metabolism, studies were undertaken of oxidative metabolism and ketogenesis by *mitochondria* obtained from the livers of rats with ethionine-induced liver injury.[8]

MATERIALS AND METHODS

Female rats of the Wistar strain were placed on diets containing 0.5 per cent ethionine and known to produce liver injury.[8] Control rats were simultaneously maintained on the same diet without ethionine and were pair-fed in amounts equal to those consumed by the ethionine-fed animals. After 21 to 35 days on the diet, the rats were fasted for 16 to 20 hours and then sacrificed by decapitation. The livers were immediately excised and homogenized in 9 parts of chilled isotonic sucrose in a glass homogenizer in the cold room at 4° C. The homogenate was centrifuged for 10 minutes at 600 × g to sediment nuclei and cellular debris. The supernate was then centrifuged at 9,000 × g for 10 minutes to precipitate the mitochondria. The mitochondrial preparation was resuspended in 2 ml. of isotonic sucrose per gram of wet weight of liver and reprecipitated at 14,000 × g for 10 minutes. The mitochondria were then resuspended in isotonic KCl, to form a suspension with a final protein concentration of 16 to 24 mg. per milliliter. Mitochondrial protein was determined, in quadruplicate, by the method of Lowry and associates.[9]

Mitochondrial oxidase activity was measured by the Warburg method. For pyruvic oxidase studies, flask contents included Na pyruvate 0.02 M, fumaric acid (neutralized with KOH) 6.7×10^{-4} M, ATP 0.001 M, $MgSO_4$ 0.002 M, DPN 2×10^{-4} M, niacinamide 0.01 M, cytochrome C 1×10^{-5} M, and phosphate 0.02 M. In determinations of alpha-keto glutaric oxidase activity, flask contents were similar to those in the above assay with the exception that pyruvate was replaced by 0.02 M alpha-keto glutaric acid (neutralized with KOH). In addition, the fumaric acid was replaced by malonic acid (neutralized with KOH) 0.005 M, in order to isolate alpha-keto glutaric oxidase activity.[1] Flask contents for butyric oxidase assay included Na butyrate 0.017 M, fumaric acid (neutralized with KOH) 6.7×10^{-4} M, KCL 0.05 M, ATP 0.001 M, $MgSO_4$ 0.002 M, cytochrome C 1×10^{-5} M, and phosphate 0.02 M. Fumaric oxidase was assayed by omitting the butyrate in the above. For succinic oxidase, contents included Na succinate 0.05 M, cytochrome 1.3×10^{-5} M, $CaCl_2$ 4×10^{-4} M, $AlCl_3$

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4×10^{-4} M, and phosphate 0.03 M. All mixtures were adjusted to pH 7.35. For all studies except succinic oxidase, mitochondrial suspension containing 8 to 12 mg. protein was added to each flask; for the latter, 3 to 5 mg. of mitochondrial protein was added per flask. The total flask volume was 3 ml., temperature: 37° C., gas phase: oxygen. Twenty per cent KOH was placed into the center wells. Specific oxidase activity was expressed as QO_2/protein, or microliters of oxygen consumed per hour per milligram of mitochondrial protein.

At the end of 30 minutes' incubation, the ketone concentration within the flask was determined by the method of Greenberg and Lester[7]; ketone production was expressed as micromoles of acetoacetic acid produced per hour per milligram of mitochondrial protein.

RESULTS

Rats fed the diet containing ethionine lost weight more rapidly than the pair-fed controls. At autopsy, the livers were grossly enlarged, pale, firm, and granular. Microscopic examination revealed lobular disarrangement, marked variation in cell size, cellular swelling, cytoplasmic coagulation, marked inflammatory cell and fibroblastic infiltration, and slight fibrosis, as previously described by Koch-Weser and Popper.[8]

The endogenous uptake of oxygen by mitochondria in the absence of substrate was negligible. In the presence of all substrates except pyruvate, a linear rate of oxygen consumption was maintained for at least 30 minutes, and this period of observation was used as a basis for calculation of hourly oxygen consumption. However, with pyruvate as substrate, there was a linear rate of oxygen consumption for more than 30 minutes only when mitochondrial preparations from control animals were utilized. When liver mitochondria from ethionine-fed rats were used, the rate declined progressively during each 10-minute period of observation. To minimize this difference, the hourly rate of oxygen consumption, with use of pyruvate as a substrate, was calculated on the basis of the uptake during the first 10 minutes following thermequilibration.

The results are shown in Table I. Marked depression of pyruvic and butyric oxidase activity was observed in the ethionine rats. The depression became even more apparent when total oxygen uptake was corrected for oxygen uptake from the 2 μM of fumarate added as a "sparker" to the flask mixtures.

TABLE I. HEPATIC MITOCHONDRIAL OXIDASE ACTIVITY*

SUBSTRATE	CONTROL NO. OF RATS	CONTROL QO_2	ETHIONINE NO. OF RATS	ETHIONINE QO_2	% DEPRESSION†	P
Pyruvate	10	67 ± 2.8	12	38 ± 2.7	43	0.001
Pyruvate (corrected for O_2 uptake from fumarate)	8	33 ± 2.4	7	12 ± 4.2	64	0.001
Butyrate	10	74 ± 3.3	12	49 ± 3.5	34	0.001
Butyrate (corrected for O_2 uptake from fumarate)	8	38 ± 1.9	7	22 ± 2.5	42	0.001
Fumarate	8	34 ± 2.0	7	29 ± 2.7	15	0.10
Succinate	15	410 ± 12	16	350 ± 17	14	0.01
Alpha-keto glutarate	8	68 ± 2.7	7	52 ± 2.8	23	0.005

*Expressed as QO_2 protein (milliliter of oxygen consumed per hour per milligram of mitochondrial protein): Mean ± S. E.

†Per cent depression $= \frac{\text{control} - \text{ethionine}}{\text{control}} \times 100$.

Depression of lesser degree in other oxidase activity was also observed in ethionine-fed animals. The depression of butyrate and pyruvate oxidation was two to three times as great as the depression of the activity of the malonate-isolated alpha-keto glutaric oxidase system in the mitochondria. Depression of succinic and fumaric oxidase activity in these mitochondria was minimal.

Ketone production by liver mitochondria from ethionine-fed rats was strikingly depressed. In these studies butyrate and pyruvate served as substrates (Table II).

TABLE II. HEPATIC MITOCHONDRIAL KETONE PRODUCTION*

SUBSTRATE	CONTROL		ETHIONINE		% DEPRESSION†	P
	NO. OF RATS	KETONES	NO. OF RATS	KETONES		
Pyruvate	9	0.63 ± 0.053	8	0.20 ± 0.027	68	0.001
Butyrate	9	0.35 ± 0.039	11	0.15 ± 0.034	57	0.001

*Expressed as micromoles acetoacetic acid per hour per milligram of mitochondrial protein: Mean ± S. E.

†Per cent depression $= \frac{\text{control} - \text{ethionine}}{\text{control}} \times 100$.

In view of the known relationship of the sulfur-containing co-factors, coenzyme A and lipoic acid (thioctic acid), to butyrate and pyruvate oxidation,[12] and because sulfur-containing amino acids appear to be essential to maintenance of morphology and function of the liver,[6, 13] studies relating to these co-factors in ethionine-induced liver injury were undertaken.

In spite of marked lipoic acid depletion of the liver mitochondria of ethionine-fed animals,[5] in vitro addition of 1 mcg. of lipoic acid failed to reverse the observed metabolic defects; injection of 60 mcg. of lipoic acid in vivo did not increase the depleted lipoic acid content of mitochondria in livers of ethionine-fed rats. In regard to coenzyme A, neither the in vitro addition of 40 units of coenzyme A* per flask nor the injection of 2,500 units of coenzyme A, 20 hours prior to sacrifice, had any effect upon the diminished oxygen consumption and ketogenesis in the presence of pyruvate and butyrate.

Choline was also studied since Artom[3] reported that the in vivo injection of choline reversed diminished butyrate oxidation observed in mitochondria of choline-depleted, fat-infiltrated rat livers. Moreover, choline is an ethionine-antagonist in vivo.[15] However, when 5 mg. of choline was injected into ethionine-fed rats, at 2 hours and at 30 minutes prior to sacrifice, no effect upon the metabolic defects could be detected. Since the flask mixtures were enriched with DPN and magnesium, the effect of these co-factors was not studied.

DISCUSSION

A marked depression of *mitochondrial* ketogenesis and oxidase activity was observed with butyrate and pyruvate substrates, indicating that the diminished ketogenesis and impaired pyruvate metabolism of experimental liver injury exists at the sub-cellular level. That this is a specific metabolic defect is sug-

*Sigma Biochemical Company.

gested by the fact that cell-free mitochondrial suspensions were used and that activity of other oxidases showed only minimal changes. If the observed differences in enzymatic activity were due to over-all replacement of functional hepatic tissue by inflammatory, necrotic, or fibrous tissue, enzyme systems tested would be expected to be proportionately affected.

In attempting to ascertain the metabolic step at which the oxidation of pyruvate and butyrate is interfered with, one should note that only a slight impairment of succinate, fumarate, and alpha-keto glutarate oxidase activities was found. This indicates that the fundamental defect responsible for the depression of oxygen consumption and ketogenesis from butyrate and pyruvate does not reside in oxidases of the Krebs cycle, but in the preceding enzymatic steps. It is known that pyruvate and butyrate must be converted to activated acetate prior to introduction into the Krebs cycle and prior to the formation of ketone bodies. It seems likely, therefore, that a failure of conversion to active acetate may be responsible for the defects described.

Depletion of known co-factors (CoA, choline, and thioctic acid) related to activation of acetate does not appear to explain the defect, since in vitro addition of these co-factors does not correct the defect. Further, the depression of pyruvic oxidase is three times as great as the depression of alpha-keto-glutaric oxidase, despite the fact that both require the same co-factors.[12]

Thus, the probable explanation for the observed depression of pyruvic and butyric oxidation and ketogenesis resides in impaired activation of acetate. This impairment may be associated either with defective synthesis of specific protein apo-enzymes or may depend upon depletion of an as yet unidentified co-factor. It is suggested that this mechanism may be responsible for the earlier observations of diminished ketogenesis and elevated pyruvic acid blood levels in patients with liver disease.

SUMMARY

Markedly diminished ketogenesis and decreased butyrate and pyruvate oxidation have been demonstrated in mitochondria obtained from the livers of rats with liver injury induced by prolonged ethionine feeding. Oxygen consumption from substrates intrinsic to the Krebs cycle showed only slight depression. The observed metabolic defects were not reversed by the in vitro or in vivo supplementation of various co-factors. The presence of a specific defect in acetate activation is suggested.

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THE OSMOTIC ACTIVITY OF THE RENAL TUBULE IN DIABETIC ACIDOSIS

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INTRODUCTION

DURING solute diuresis in normal hydropenic man, the osmolarity of the urine decreases to approach asymptotically that of plasma.[1, 2] Furthermore, the urine flow is correlated with the magnitude of the excreted load of solutes regardless of the nature of the solutes.[2]

The patient with diabetic acidosis resembles this experimental subject in that he is hydropenic and is undergoing solute diuresis with glucose derived from endogenous sources. Data are here reported dealing with the relations of urinary flow, osmolarity, and load in patients with ketoacidosis.

METHODS

Nineteen subjects with diabetic acidosis were selected for study. Of these, 14 formed a group of moderate severity. They were of ages 20 to 40, drowsy or stuporous, and free from shock, oliguria, and gross complications. The initial values of plasma urea nitrogen and carbon dioxide content were less than 30 mg. per cent and 14 mM per liter, respectively. All recovered uneventfully. Two of these subjects were studied during two admissions each for acidosis. The remaining 5 patients on whom observations were made had severe acidosis complicated by conditions such as full coma, shock, or oliguria, with recovery in 2 only.

Urine specimens were collected before and immediately after the institution of therapy for ketoacidosis. Periods of collection ranged from 30 to 60 minutes with some of 180 minutes. In 5 instances 250 ml. of 10 per cent glucose was administered intravenously to increase urine flow. Therapy consisted of conventional schedules of subcutaneous insulin and intravenous saline. Specimens collected after the onset of treatment were obtained before insulin action was considered significant and before any degree of hydration occurred. Blood specimens were collected at the midpoints of periods of urine collections.

In order to compare the relations of flow and osmotic activity in acidosis and recovery, 5 of the subjects were studied again several weeks later when in normal state. These subjects were rendered hydropenic by water deprivation for 14 hours and then given glucose by mouth and 50 per cent glucose intravenously. Insulin was withheld until the tests were completed. Preliminary and loading urine specimens were obtained over 20-minute periods, with use of a catheter in the female. Blood specimens were obtained at the midpoints of periods.

Determinations were made of glucose,[3] urea nitrogen,[4] and CO_2 content.[5] Sodium and potassium were determined by flame photometry with use of a Weichselbaum-Varney flame photometer, and total urinary osmolality by freezing point depression.

Rates of urine flow were corrected to 1.73 square meter body surface area. The rate of total solute excretion is designated the total load, the product of the rate of urine flow and the urine osmolarity, and is expressed in mOsm./min./1.73 square meter. The rate

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of water reabsorption from the isosmotic solute or osmolar clearance was calculated as $\frac{\text{total load} \times 1{,}000}{\text{plasma osmolarity}}$—urine flow. This moiety has been designated the water economy by some[6] and T_cH_2O by others.[7] Plasma osmolarity was estimated as 2 (Na + K) − 8 + glucose +urea.[8] In these concentrations, osmolarity and osmolality are considered equivalent.

In the analysis of data, student's "t" test was used to determine the significance of single regression coefficients, the difference between two regression coefficients, and the difference between mean values of Y^I and Y^{II} at a given value of X. In the third test the sampling variance of the estimate of the mean value of Y calculated from the regression was determined from the sampling variance of the mean of Y and the sampling variance of X for values other than the mean. Observed correlations were tested for significance of difference by the Z test of Fisher.

RESULTS

In Tables I and II are listed the values of urinary flow, osmolarity, and total and glucose loads during acidosis and recovery, respectively. In Table III are given data used to derive water economy.

TABLE I. URINE FLOW AND COMPOSITION DURING DIABETIC ACIDOSIS. ALL VALUES ARE CORRECTED TO 1.73 M^2 BODY SURFACE AREA

PATIENT	PERIOD	FLOW (ML./MIN.)	TOTAL SOLUTES		GLUCOSE	
			mOsm./L.	mOsm./MIN.	mOsm./L.	mOsm./MIN.
Moderate Acidosis						
WJ_1	1	4.09	727	2.97	308	1.26
	2	5.02	684	3.43	364	1.83
HB	1	3.29	432	1.42	92	0.30
JW	1	2.66	636	1.69	160	0.42
	2	3.49	615	2.15	191	0.67
LA	1	1.45	525	0.76	222	0.32
	2	2.86	510	1.46	202	0.58
MA	1	2.51	451	1.13	199	0.50
	2	5.22	485	2.53	228	1.19
RW	1	2.83	461	1.30	199	0.56
	2	6.51	484	3.15	255	1.66
WJ_2	1	3.56	711	2.53	246	0.88
	2	2.99	678	2.03	342	1.02
CR	1	1.94	500	0.98	283	0.56
	2	2.34	519	1.21	286	0.66
VH	1	5.41	472	2.55	179	0.97
	2	6.36	451	2.87	168	1.07
MR	1	4.07	564	2.30	198	0.81
	2	7.00	573	4.01	295	2.07
JL_1	1	2.94	608	1.79	190	0.56
	2	6.03	570	3.44	214	1.29
MP	1	1.67	634	1.06	228	0.38
AM	1	1.41	478	0.67	154	0.22
	2	3.57	458	1.64	128	0.46
LS	1	1.55	497	0.77	119	0.18
	2	4.81	514	2.47	134	0.65
JL_2	1	2.60	800	2.08	229	0.60
	2	4.94	672	3.32	379	1.87
JH	1	2.98	517	1.54	225	0.67
	2	6.39	518	3.25	281	1.80
Mean (30)		3.75	558	2.09	223	0.87
Severe Acidosis						
HD	1	4.38	382	1.67		
CB	1	2.56	419	1.07		
NS	1	0.15	399	0.06		
MS	1	0.33	555	0.18		
	2	0.18	400	0.07		
GR	1	0.04	532	0.02		

TABLE II. URINE FLOW AND COMPOSITION DURING GLUCOSE DIURESIS IN DIABETIC SUBJECTS AFTER RECOVERY FROM DIABETIC ACIDOSIS. 0 SIGNIFIES PRELOADING PERIOD. ALL VALUES ARE CORRECTED TO 1.73 M^2 BODY SURFACE AREA.

PATIENT	PERIOD	FLOW (ML./MIN.)	TOTAL SOLUTES	
			mOsm./L.	mOsm./MIN.
MP	0	0.61	864	0.53
	1	1.92	850	1.63
	2	2.40	858	2.06
	3	4.28	758	3.24
	4	7.49	683	5.12
LA	0	0.31	1,021	0.32
	1	1.19	1,037	1.23
	2	1.62	828	1.34
	3	3.67	695	2.55
LS	0	0.76	792	0.60
	1	2.30	661	1.52
	2	4.07	599	2.44
	3	5.35	569	3.04
	4	6.30	564	3.55
	5	7.13	569	4.06
MR	0	0.60	1,206	0.72
	1	2.23	983	2.19
	2	1.59	940	1.49
	3	2.61	860	2.24
	4	2.06	951	1.96
	5	2.45	923	2.26
JL	4	1.01	754	0.76
	5	2.87	635	1.82
	6	4.56	652	2.97
	7	4.39	655	2.88
Mean of loading periods (21)		3.40	763	2.40

TABLE III. WATER ECONOMY IN ACIDOSIS AND RECOVERY. 0 SIGNIFIES PRELOADING PERIOD. ALL VALUES ARE CORRECTED TO 1.73 M^2 BODY SURFACE AREA

PATIENT	PERIOD	URINE		PLASMA					H_2OE (ML./MIN.)
		FLOW (ML./MIN.)	LOAD (mOsm./MIN.)	GLUCOSE (mOsm./L.)	UREA (mOsm./L.)	NA (mOsm./L.)	K (mOsm./L.)	OSM. ACT. (mOsm./L.)	
Acidosis									
LA	1	1.45	0.76	37	8	132	8	317	0.95
MR	1	4.07	2.30	20	5	144	5	315	3.23
MP	1	1.67	1.06	32	10	131	7	310	1.75
LS	1	1.55	0.77	30	7	146	6	333	0.76
Recovery									
LA	0	0.31	0.32	7	6	143	4	299	0.76
	1	1.19	1.23	8	6	144	4	302	2.88
	2	1.62	1.34	19	7	140	4	306	2.76
MR	0	0.60	0.72	18	9	138	4	303	1.78
	2	1.59	1.49	22	9	139	4	309	3.23
	4	2.06	1.96	23	8	137	4	305	4.37
MP	0	0.61	0.53	12	5	144	4	305	1.13
	1	1.92	1.63	19	5	147	5	320	3.17
	4	7.49	5.12	35	5	144	5	330	8.03
LS	0	0.76	0.60	17	6	140	4	303	1.22
	2	4.07	2.44	33	7	135	4	310	3.80
	3	5.35	3.04	39	7	136	4	318	4.21

Osmotic Activity.—In Fig. 1 are presented the values of urinary osmotic activity and flow in all the patients with moderate ketoacidosis. The osmotic

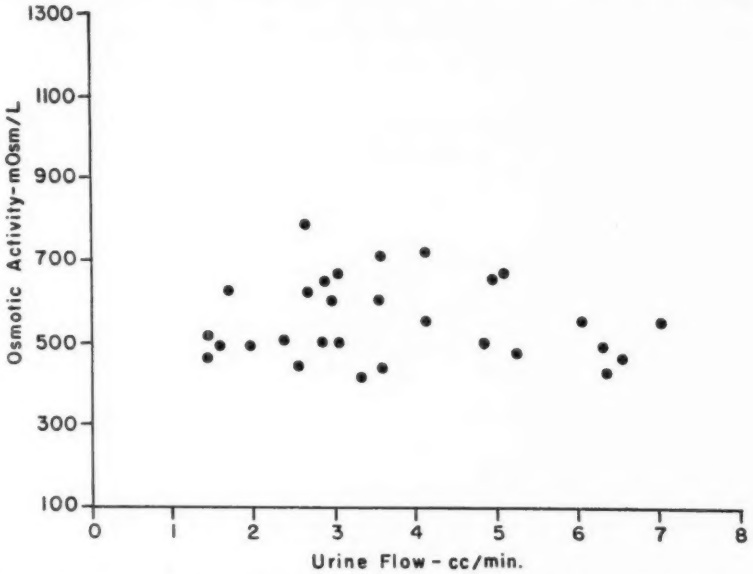

Fig. 1.—The effective osmotic activity of the urine in moderately severe diabetic acidosis.

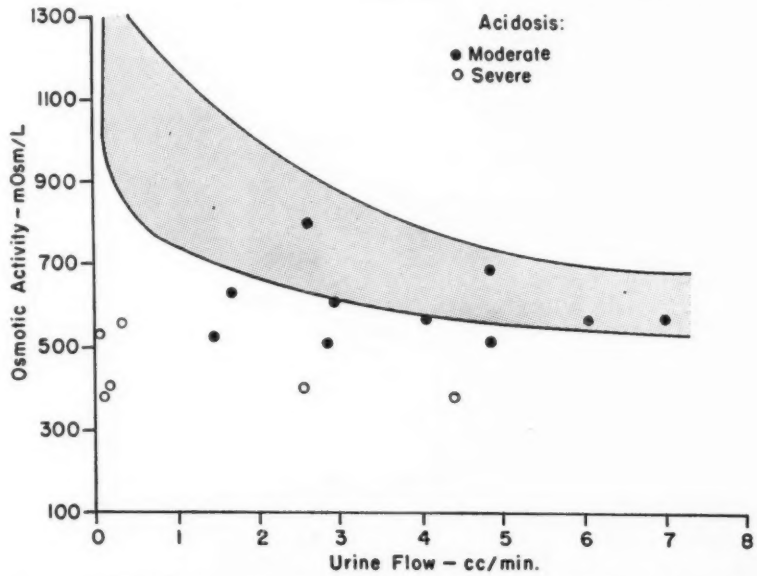

Fig. 2.—The effective osmotic activity of the urine in 5 patients studied in acidosis and after recovery. The shaded area represents all values (25) observed in the recovery state. The closed circles represent the values observed in the same patients in the acidotic state. The open circles represent the values observed in 5 other patients with severe complicated acidosis.

activity varied from 432 to 800 mOsm. per liter with a mean value of 558 mOsm. per liter. No relationship is apparent between osmolarity and flow. In

Fig. 2 are given the data obtained in the 5 patients studied during acidosis and recovery. In the normal state the osmolarity-flow relation was similar to that noted by others.[1, 2] However, in acidosis the values tended to fall slightly below the recovery data, suggesting some impairment in osmotic activity. For contrast are shown also the data obtained in the severely ill patients

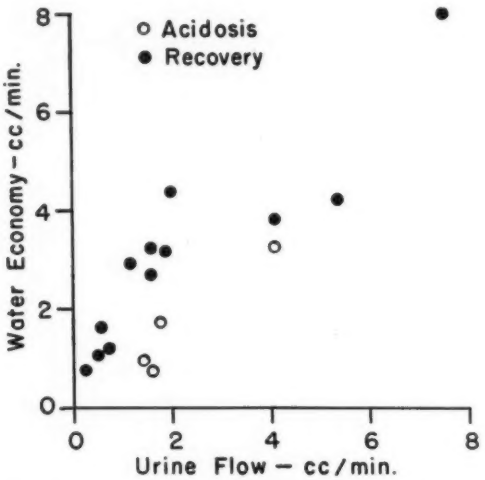

Fig. 3.—The values of water economy observed in 4 patients with moderately severe acidosis obtained during acidosis and after recovery.

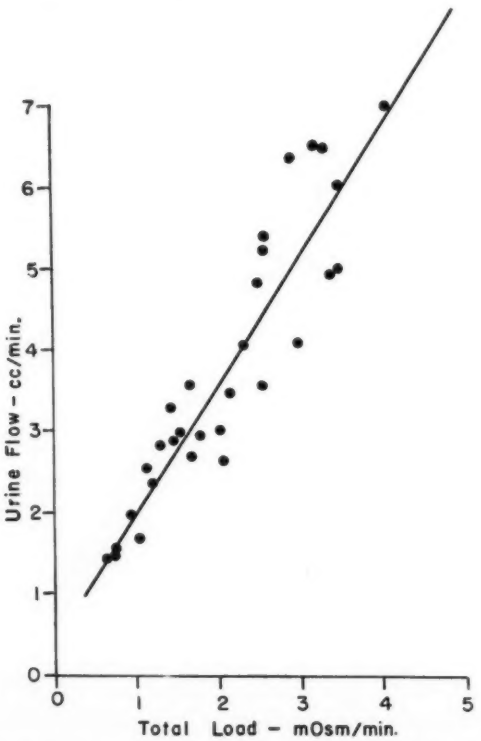

Fig. 4.—The relation of urine flow to total load in moderately severe acidosis.

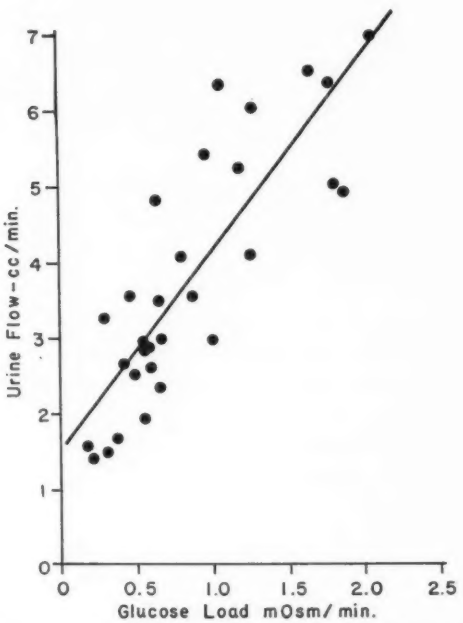

Fig. 5.—The relation of urine flow to glucose load in moderately severe acidosis.

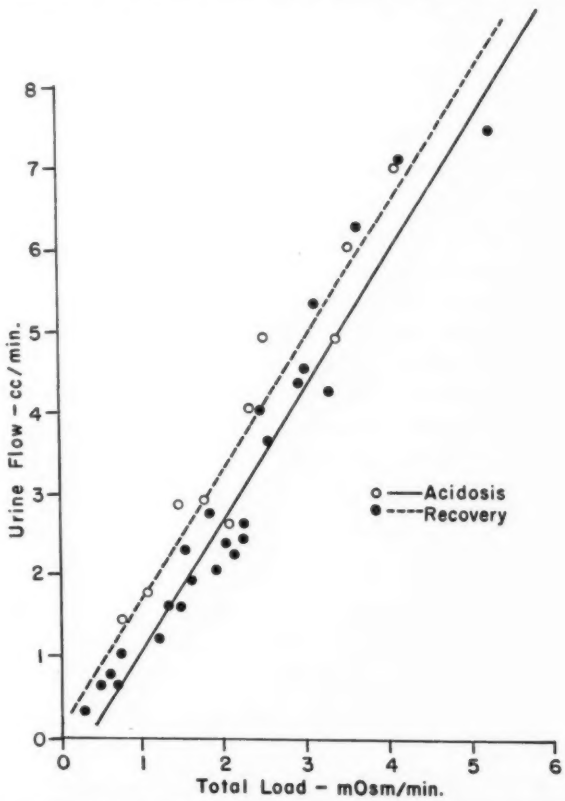

Fig. 6.—The relation of urine flow to total load in 5 patients with moderately severe acidosis studies during acidosis and after recovery.

with vascular collapse. Even at very low flows the urinary osmolarity approached that of plasma. In Fig. 3 water economy is plotted against urine flow in 4 patients studied in acidosis and recovery. Granting that the data are few, they suggested some diminution of distal tubular water reabsorption in acidosis.

Flow-Load Relations.—In Fig. 4 are shown the values of urinary flow and total load in the 16 instances of moderate ketoacidosis. The relation is linear and significant ($T = 12.7$), showing that the flow is closely related to the total solute load destined for excretion. Flow is plotted against glucose load in Fig. 5. Again there is a significant relation ($T = 8.32$). Although the degree of scatter of the flow-glucose load curve appears greater than that of the flow-total load curve, statistical analysis revealed no significant difference ($P < 0.1$). In Fig. 6 the flow-load relations of the 5 patients studied in acidosis and recovery are given. There is a slight but significant distance between the curves, suggesting that uncomplicated moderate ketoacidosis causes only minimal impairment of concentrating ability ($P < 0.01$).

DISCUSSION

The absence of a relation between urinary osmotic activity and flow in moderate ketoacidosis is not surprising in view of the many variables which may affect renal tubular function in this condition. For example, there is evidence that the concentrating function of the kidney is fundamentally measured by the osmotic urinary-plasma ratio.[9] Accordingly, the dispersion of plasma osmolarities resulting from the different glucose concentrations may partially account for the scatter. In addition, other factors such as potassium deficiency[10] or renal ischemia[11] may alter tubular function. Nevertheless, from the data reported herein it is apparent that the concentrating ability of the kidney was appreciably decreased only when hypotension or other complication was present.

The diuresis of diabetic acidosis has been attributed to hyperglycemia and glycosuria.[12] However, in the present study no relation was demonstrable between the concentration of plasma glucose and urine flow. It has been suggested that urine flow is related to glucose load,[13] and to total load.[14] Despite the lack of significant difference between the degrees of scatter of the curves of flow versus total load and flow versus glucose load, it seems likely that total load is the determining factor. As glucose comprised the largest and a relatively constant fraction (40 per cent) of total load, the glucose load would of necessity influence total load and flow.

SUMMARY

Observations made on patients with diabetic acidosis revealed no correlation between urinary total osmolarity and flow and the fact that osmotic activity is minimally diminished in the uncomplicated case. The flow is closely related to the total load and to glucose load as well, since glucose comprises the largest and a relatively constant constituent of the load.

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METABOLIC EFFECTS OF FAT EMULSIONS ADMINISTERED INTRAVENOUSLY TO HUMAN SUBJECTS

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THE potential value of intravenous fat emulsions in the nutritional management of patients has been emphasized on many occasions.[1-3] Although utilization of emulsified fat given intravenously has been demonstrated in experimental animals,[4, 5] studies of the metabolic effects of intravenous fat in man have been limited because of difficulties in obtaining fat emulsions consistently free of side effects.[6]

Previous reports on the metabolic effects of intravenous fat in man have indicated that under suitable nutritional conditions, fat given by the intravenous route is utilized for calories and decreases nitrogen and potassium deficits induced by oral intakes inadequate in caloric content.[7, 8] Although such reports have been encouraging, relatively few controlled studies involving intravenous fat administration to human subjects have been performed, and therefore the need for further assessment of the nutritional value of fat given intravenously has been urgent.

In this study, metabolic effects of fat emulsions administered intravenously to four healthy volunteer subjects on oral intakes inadequate in calories are described.

MATERIALS AND METHODS

1. *Materials.*—The fat emulsions used in these experiments had the following composition per 100 ml.: olive oil 15 Gm., purified phosphatide 1 Gm., Pluronic F-68† 0.3 Gm., and dextrose 4.5 Gm.‡ The caloric content per liter was approximately 1,600.

2. *Methods.*—Representative portions of diets were homogenized and analyzed for their content of nitrogen and potassium. Feces were prepared similarly and analyzed. Urinary potassium and nitrogen were measured directly. All determinations were made in duplicate on aliquot samples.

Total nitrogen was determined by a macro-Kjeldahl method. Potassium was determined by means of a flame photometer, the fecal and dietary aliquot samples having been prepared by a modification of the wet-ashing method described by Peters and Van Slyke.[10]

Caloric intake was calculated from weighed portions and refusals on the basis of standard tables.[11] A Toledo scale accurate to 50 grams was used to measure body weight. Subjects were weighed every morning prior to the first meal and after voiding.

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†Prepared by condensing ethylene oxide with a hydrophobic base formed by the condensation of propylene oxide with propylene glycol. Wyandotte Chemicals Corporation, Wyandotte, Mich.

‡The emulsions were prepared by The Upjohn Company, essentially according to a procedure described by Geyer and associates, and were furnished through the courtesy of Dr. Robert W. Heinle.

3. *Subjects.*—Four male graduate students served as subjects. All of them were studied in the hospital. They remained ambulatory but were limited to light activity. All of the subjects were healthy and gave no history of recent illness. Each subject had been instructed to take a preparatory diet low in protein for three days prior to admission to the hospital. Data on the experimental subjects, including their estimated caloric expenditure while under study, are in Table I.

TABLE I. DATA ON EXPERIMENTAL SUBJECTS

SUBJECTS	AGE	HEIGHT (CM.)	WEIGHT (KG.)	ESTIMATED CALORIC EXPENDITURE[12] (CAL./DAY)
R. M.	27	174.6	79.5	2,500-2,700
M. J.	24	185.0	65.2	2,300-2,500
C. L.	22	173.0	53.1	2,100-2,300
J. C.	24	185.0	70.1	2,400-2,600

4. *Plan.*—All four of the experiments began with a control period (Period I) lasting 5 to 7 days, during which a constant diet inadequate in calories and adequate in protein was consumed. The control period was followed by an experimental period (Period II) lasting 5 to 6 days, during which the caloric intake was supplemented by fat emulsions administered intravenously. During Period II the oral diet remained constant except that the intake of carbohydrate was reduced by approximately the amount provided by the intravenous fat emulsion on any given day.

In three of the experiments, the period of caloric supplementation with intravenous fat was followed by a second control period (Period III) lasting 5 to 7 days, which was similar to Period I.

In two of the experiments, the second control period was followed by another period (Period IV) lasting 6 days, during which (oral) fat was added to the basic constant diet in amounts comparable to what was provided by the intravenous fat emulsions during Period II.

RESULTS

A statistical comparison of the mean nitrogen and potassium balances for all control and experimental periods is in Table II. The details of each individual experiment are given in Tables III and IV, and Figs. 1 and 2.

Experiment 1 (Fig. 1).—During Period I when the caloric and nitrogen intakes were restricted, there was a persistently negative nitrogen balance averaging –3.8 ± 0.9 Gm.* per day. The potassium balance also was negative during the first period, averaging –22.6 ± 9.5 meq. per day. The K:N excretion ratio (milliequivalents of potassium to grams of nitrogen) for this period was approximately 5.9. The subject's weight fell steadily from 79.5 kilograms to 76.5 kilograms (average per day –0.4 kilogram).

During Period II while the intravenous fat was being administered, the nitrogen deficit diminished markedly, averaging only 0.6 ± 1.9 Gm. per day. The potassium balance became positive, averaging 1.6 ± 12.1 meq. per day. The rate of weight loss decreased to an average of 0.2 kilogram per day.

Experiment 2 (Table III).—During the first period, nitrogen balances were negative, averaging –3.6 ± 1.2 Gm. per day. The average potassium balance was –11.8 ± 10.2 meq. per day, and average weight loss was 0.22 kilogram per day. Rates of potassium and weight loss diminished toward the end of the period.

*Standard Deviation $= \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$

TABLE II. STATISTICAL COMPARISON OF MEAN NITROGEN AND POTASSIUM BALANCES IN FOUR NORMAL SUBJECTS GIVEN FAT EMULSIONS INTRAVENOUSLY

EXPERIMENT	PERIOD	DAYS OF PERIOD	ORAL CALORIES (AV./DAY)	CALORIES FROM FAT SUPPLEMENT (AV./DAY)	MEAN NITROGEN INTAKE (GM./DAY)	MEAN NITROGEN BALANCE (GM./DAY)	DIFFERENCE BETWEEN COMPARED MEANS	p	MEAN POTASSIUM INTAKE (MEQ./DAY)	MEAN POTASSIUM BALANCE (MEQ./DAY)	DIFFERENCE BETWEEN COMPARED MEANS	p
1. R. M.	I	7	1,000	0	9.4	-3.8 ± 0.9	3.2	<0.01	46.9	-22.6 ± 9.5	24.2	<0.01
	II	6	1,000	1,451*	9.4	-0.6 ± 1.9			46.9	1.6 ± 12.1		
2. M. J.	I	5	981	0	9.9	-3.6 ± 1.2	2.9	<0.02	32.1	-11.8 ± 10.2	18.7	<0.01
	II	6	981	1,425*	9.9	-0.7 ± 1.7			32.1	6.9 ± 7.8		
	III	5	981	0	9.9	-2.4 ± 1.6	1.7	N.S.‡	32.1	-10.9 ± 7.5	17.8	<0.01
	IV	6	981	1,350†	9.9	0.1 ± 2.1	2.5	N.S.‡	32.1	-21.2 ± 14.2	10.3	N.S.‡
3. C. L.	I	5	950	0	9.0	-2.7 ± 1.5	1.8	<0.05	49.6	-9.4 ± 6.0	26.8	<0.01
	II	6	950	1,176*	9.0	-0.9 ± 1.0			49.6	17.4 ± 7.2		
	III	7	950	0	9.0	-1.2 ± 1.4	0.3	N.S.‡	49.6	-2.8 ± 15.4	20.2	<0.02
	IV	6	950	1,199†	9.0	-0.3 ± 1.8	0.9	N.S.‡	49.6	7.4 ± 16.3	10.2	N.S.‡
4. J. C.	I	5	1,000	0	9.6	-4.0 ± 1.0	1.4	N.S.‡	48.3	-6.9 ± 6.2	3.8	N.S.‡
	II	5	1,000	1,431*	9.6	-2.6 ± 1.4			48.3	-3.1 ± 15.5		
	III	7	1,000	0	9.6	-1.8 ± 1.3	0.8	N.S.‡	48.3	1.1 ± 2.1	4.2	N.S.‡

*Intravenous fat.
†Oral fat.
‡Not significant ($p > 0.05$).

TABLE III. COMPARISON OF METABOLIC EFFECTS OF ORALLY AND INTRAVENOUSLY ADMINISTERED FAT IN A NORMAL SUBJECT (M. J.) ON A CALORICALLY DEFICIENT DIET

DATE	BODY WEIGHT (KG.)	CALORIES		NITROGEN (GM.)				POTASSIUM (MEQ.)			
		TOTAL CALORIES	FROM FAT SUPPLEMENT	ORAL INTAKE	URINE	STOOL	BALANCE	ORAL INTAKE	URINE	STOOL	BALANCE
6/23	66.00	981	–	9.9	12.1	–	-2.2	32.1	53.3	–	-21.2
6/24	65.24	981	–	9.9	13.1‡	2.4	-5.5	32.1	34.1	18.2	-20.2
6/25	64.77	981	–	9.9	13.1‡	–	-3.2	32.1	47.7	0.3	-15.9
6/26	64.88	981	–	9.9	13.3‡	–	-3.4	32.1	30.2	0.4	+1.6
6/27	64.90	981	–	9.9	13.3‡	0.1	-3.5	32.1	34.5	1.0	-3.4
6/28	65.02	1,777	777*	9.9	11.9	–	-2.0	32.1	30.3	–	-1.8
6/29	64.89	2,244	1,244*	9.9	10.9	0.1	-1.1	32.1	26.0	0.9	+5.3
6/30	64.70	2,555	1,555*	9.9	10.2	–	-0.3	32.1	22.0	–	+10.1
7/1	64.66	2,555	1,555*	9.9	11.0	1.7§	-2.8	32.1	22.4	14.9§	-5.2
7/2	64.54	2,555	1,555*	9.9	7.8	–	+2.1	32.1	16.0	–	+16.1
7/3	64.48	2,865	1,865*	9.9	9.8	–	-0.1	32.1	19.1	–	+13.0
7/4	64.69	1,000	–	9.9	10.8	–	-0.9	32.1	38.3	–	-6.1
7/5	64.53	1,000	–	9.9	12.6	–	-2.7	32.1	35.5	–	-3.4
7/6	64.25	1,000	–	9.9	11.9	–	-2.0	32.1	40.4	–	-8.3
7/7	63.75	1,000	–	9.9	14.9	–	-5.0	32.1	47.2	–	-15.1
7/8	63.61	1,000	–	9.9	11.3	0.1	-1.5	32.1	53.3	0.7	-21.8
7/9	63.30	2,350	1,350†	9.9	11.9	1.3	-3.3	32.1	59.8	10.7	-38.3
7/10	63.10	2,350	1,350†	9.9	7.1	–	+2.8	32.1	48.4	–	-16.3
7/11	63.10	2,350	1,350†	9.9	9.6	–	+0.3	32.1	53.0	–	-20.8
7/12	63.13	2,350	1,350†	9.9	8.9	2.4	-1.4	32.1	53.8	19.1	-40.7
7/13	62.92	2,350	1,350†	9.9	8.8	–	+1.1	32.1	39.3	–	-7.2
7/14	62.67	2,350	1,350†	9.9	8.6	0.2	+1.1	32.1	34.1	1.2	-3.2

*Intravenous fat.
†Oral fat.
‡2-day pool due to incorrect collecting.
§Enema.

TABLE IV. METABOLIC FINDINGS IN A NORMAL SUBJECT (J. C.) GIVEN FAT EMULSIONS INTRAVENOUSLY TO SUPPLEMENT A CALORICALLY DEFICIENT DIET

DATE	BODY WEIGHT (KG.)	CALORIES		NITROGEN (GM.)				POTASSIUM (MEQ.)			
		TOTAL CALORIES	FROM I.V. FAT	ORAL INTAKE	URINE	STOOL	BALANCE	ORAL INTAKE	URINE	STOOL	BALANCE
7/16	70.10	1,000	–	9.6	13.0	1.3	-4.7	48.2	49.8	11.6	-13.2
7/17	69.50	1,000	–	9.6	13.9	–	-4.3	48.2	56.8	–	-8.6
7/18	69.40	1,000	–	9.6	14.8	–	-5.2	48.2	50.0	–	-1.8
7/19	69.65	1,000	–	9.6	12.7	–	-3.1	48.2	47.6	–	+0.6
7/20	69.10	1,000	–	9.6	11.6	0.8	-2.8	48.2	51.5	9.2	-12.5
7/21	68.45	1,933	933	9.6	11.1	–	-1.5	48.2	56.4	–	-8.2
7/22	68.10	2,555	1,555	9.6	10.6	–	-1.0	48.2	27.2	–	+21.0
7/23	68.00	2,555	1,555	9.6	10.2	3.9	-4.5	48.2	32.2	37.8	-21.8
7/24	67.80	2,555	1,555	9.6	10.0	2.5	-2.9	48.2	31.7	19.9	-3.4
7/25	67.50	2,555	1,555	9.6	9.8	2.8	-3.0	48.2	31.7	19.9	-3.4
7/26	67.40	1,000	–	9.6	9.1	–	+0.5	48.2	49.4	–	-1.2
7/27	66.55	1,000	–	9.6	10.6	–	-1.0	48.2	44.9	–	+3.4
7/28	66.70	1,000	–	9.6	11.4	–	-1.8	48.2	45.0	–	+3.2
7/29	66.65	1,000	–	9.6	12.0	–	-2.4	48.2	46.1	–	+2.1
7/30	66.40	1,000	–	9.6	12.4	1.1	-3.9	48.2	42.2	7.9	-1.9
7/31	66.00	1,000	–	9.6	10.8	–	-1.2	48.2	48.2	–	0.0
8/1	65.25	1,000	–	9.6	12.1	–	-2.5	48.2	46.0	–	+2.2

During Period II the nitrogen deficit decreased to an average of 0.7 ± 1.7 Gm. per day. The potassium balance was positive, averaging 6.9 ± 7.8 meq. per day. Weight loss was negligible, averaging 0.04 kilogram per day.

During the third period, after discontinuance of the intravenous fat, the subject's nitrogen deficit progressively increased (average 2.4 ± 1.6 Gm. per day). The potassium balance again became negative (average –10.9 ± 7.5 meq. per day). The rate of weight loss increased to an average of 0.28 kilo-

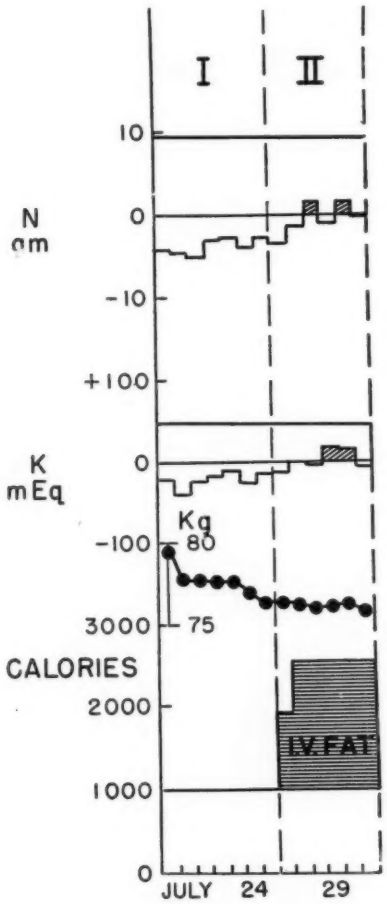

Fig. 1.—(R. M.) Decrease in nitrogen and potassium deficits following intravenous fat administration in a normal subject on a low caloric intake.

Nitrogen and potassium intakes are charted upward from the zero line, and outputs are charted downward from the top of the intake line. An excess of output over intake is seen as an area below the zero line: if output is less than intake, the difference is represented by a shaded area above the zero line.

gram per day. In Period IV, while the supplementary oral fat was being administered, the nitrogen balance became positive (average 0.1 ± 2.1 Gm. per day). The potassium loss was not appreciably changed (average –21.2 ± 14.2 meq. per day).

Experiment 3 (Fig. 2).—During Period I nitrogen balances were negative, averaging –2.7 ± 1.5 Gm. per day. Potassium balances also were negative, averaging –9.4 ± 6.0 meq. per day. The K:N deficit ratio was 3.0. The subject's weight fell from 53.1 to 52.0 kilograms; accordingly, the average weight loss per day was 0.22 kilogram.

During Period II, while the intravenous fat was being administered, the nitrogen deficit diminished, averaging 0.9 ± 1.0 Gm. per day. For the three days that the intravenous fat provided 1,500 extra calories per day, the nitrogen balance averaged 0.07 ± 0.41 Gm. per day.

The potassium balance during Period II became positive (mean 17.4 ± 7.2 meq. per day). Body weight remained constant at 52.5 kilograms.

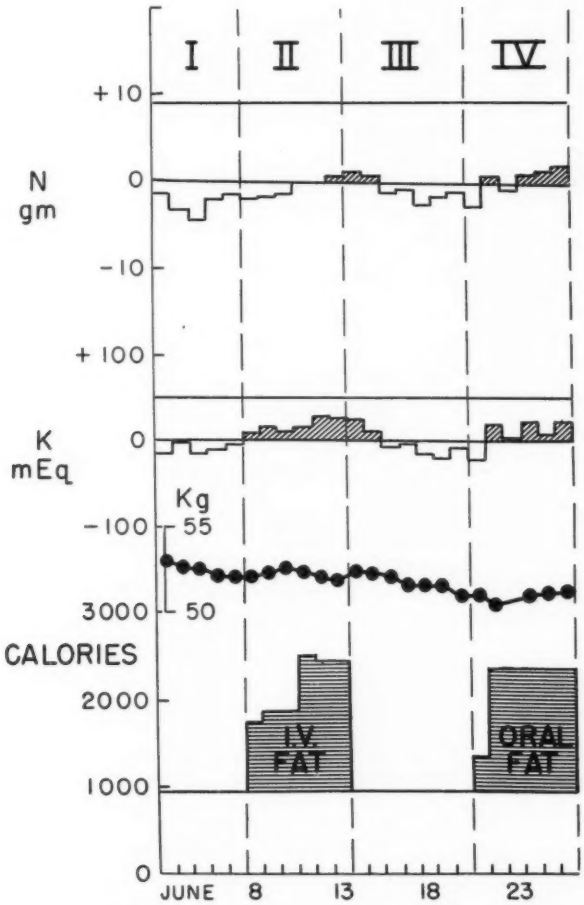

Fig. 2.—(C. L.) Comparison of intravenously administered fat and supplementary oral fat in terms of their respective effects on nitrogen and potassium balances in a subject on a calorically deficient diet.

During Period III, after discontinuance of the intravenous fat, nitrogen and potassium excretion increased until appreciable negative nitrogen and potassium balances were measured at the end of the period. Average nitrogen and potassium deficits were 1.2 ± 1.4 Gm. and 2.8 ± 15.4 meq. per day, respectively. Weight loss again occurred (average 0.18 kilogram per day).

Period IV was characterized by reappearance of positive nitrogen and potassium balances coincident with the increase in the calorie intake provided

by the oral fat supplement. The nitrogen balance averaged –0.3 ± 1.8 Gm. per day, and the potassium balance averaged 7.4 ± 16.3 meq. per day. Weight was gained at an average rate of 0.04 kilogram per day.

Experiment 4 (Table IV).—During the first period, nitrogen balances were always negative and the nitrogen deficit averaged 4.0 ± 1.0 Gm. per day. Potassium balances were minimally negative, averaging –6.9 ± 6.2 meq. per day. Body weight declined from 70.1 kilograms to 68.5 kilograms, an average loss of 0.3 kilogram per day.

In Period II the nitrogen deficit decreased, averaging 2.6 ± 1.4 Gm. per day. Excretion of urinary nitrogen diminished markedly, but on the third, fourth, and fifth days of Period II, the subject passed diarrheal stools which increased the total nitrogen output on these days by 2.9 Gm. nitrogen per day. The potassium deficit also decreased during Period II, averaging 3.1 ± 15.5 meq. per day. Again urinary potassium excretion diminished appreciably while the intravenous fat supplementation was in progress, but considerable potassium was lost during the three days of diarrhea, augmenting the potassium deficits by an average of 25.9 meq. per day on those days.

During the third period after intravenous fat had been discontinued, the subject's average nitrogen deficit was less than it was in Period II (–1.8 ± 1.3 Gm. per day). Urinary nitrogen excretion increased, compared with Period II, but the subject passed only one stool (containing 1.4 Gm. nitrogen) in the seven-day period. Total nitrogen deficit tended to increase as Period III progressed. Potassium balance in Period III averaged 1.1 ± 2.1 meq. per day and showed no trend.

DISCUSSION

In Experiment 1, fat emulsions were administered intravenously as a caloric supplement to a patient on a diet low in calories and "adequate" in protein. During the control period while the calorie intake was grossly inadequate, nitrogen and potassium deficits were appreciable. During the period of caloric supplementation with intravenous fat, the nitrogen deficit diminished markedly and a positive potassium balance was achieved. These changes were statistically significant.

In Experiments 2 and 3, similar observations with respect to the effect of intravenous fat on nitrogen and potassium balances were made on subjects on calorically inadequate diets. Again, when fat was administered intravenously as a caloric supplement, nitrogen and potassium deficits were either abolished or greatly diminished. Also, in these two experiments, an attempt was made to compare intravenously administered fat emulsions with orally administered fat supplements in terms of their respective effects on nitrogen and potassium balances in the same subject. Thus, Experiments 2 and 3 were planned with two additional periods; a second "control" period (Period III), during which no fat supplement was administered, and a second "experimental" period (Period IV), during which the dietary fat intake was increased by an amount comparable to the amount contributed by intravenously administered fat in the first experimental period (Period III).

During the period of administration of intravenous fat emulsion, nitrogen deficits were virtually abolished and the previously negative potassium balances became positive. Again, these changes were statistically significant. During the second control period in both experiments, there was a trend in the direction of increasing nitrogen and potassium deficits, but such deficits were less than those observed during the first control period. Previous studies with intravenous fat in animals[5, 12] and in man[8] suggest that such preparations may continue to be metabolized for a variable period of time after administration of them is complete. Therefore, the nitrogen and potassium-sparing effects of the intravenous fat may have extended well into the second control period. Probably for this reason (and also because nitrogen and potassium excretion rates tend to diminish spontaneously during the early phases of undernutrition[14]), less nitrogen and potassium were lost during the second control period than were lost during the first control period.* Thus, when potassium and nitrogen balances in the second control period are compared with potassium and nitrogen balances in either of the two experimental periods, the differences between the compared means, with one exception, are not statistically significant.

When oral fat was added to the basic diet during Period IV (Experiments 2 and 3), in an amount equivalent to that provided intravenously in Period II, the "sparing" effects on nitrogen balance were similar.

In the second experiment (Subject M. J.), however, intravenous fat administration was associated with a mean positive potassium balance of 6.9 ± 7.8 meq. per day, while oral fat administration was associated with a mean potassium deficit of 21.2 ± 14.2 meq. per day. In the third experiment (Subject C. L.), potassium balances became positive both when intravenous fat and when oral fat supplements were being given, although the rate of potassium retention when intravenous fat was administered was somewhat greater. The unanticipated negative potassium balance which was measured in Period IV of the second experiment remains unexplained.

Of particular interest were the striking changes in potassium balance which took place in the first three experiments when intravenous fat was administered. The potassium balances responded more dramatically to intravenous fat administration than did the nitrogen balances. This finding of a greater sensitivity of the potassium response is consistent with results obtained in earlier balance studies in man involving administration of intravenous fat emulsions.[8]

In Experiment 4, negative nitrogen and potassium balances occurred during the first control period, but the slight decrease of the deficit which was associated with intravenous fat supplementation was not statistically significant. Unfortunately, the subject developed diarrhea during Period II, and the augmentation of the nitrogen and potassium losses which ensued (see

*When the means of the nitrogen and potassium balances for Periods I and III (Experiments 2 and 3) are compared, they do not differ significantly (t-test).

Table III) masked any significant changes which might have taken place. Similarly, marked constipation which occurred during the second control period made interpretation of the results difficult.

The findings in the first three experiments indicate that the fat emulsions which were administered intravenously must have been used effectively for caloric purposes, since the nitrogen and potassium deficits associated with calorically inadequate diets were significantly reduced during the periods when the intravenous fat was being given. The effects on nitrogen and potassium balances obtained when intravenous fat was administered were generally comparable to those which were observed when the same amount of supplementary fat was administered orally.

SUMMARY

Studies on the metabolic effects of 15 per cent fat emulsions administered intravenously to four healthy volunteer subjects maintained on constant diets inadequate in calories and adequate in protein are described.

In three of the subjects, the negative nitrogen and potassium balances produced by caloric privation were significantly decreased when intravenous fat emulsions providing approximately 1,200 to 1,400 calories per day were administered as caloric supplements. In every instance, the potassium balances responded more dramatically to intravenous fat administration than did the nitrogen balances.

Two of the subjects were also given supplementary oral fat in amounts similar to the amounts supplied by the intravenously administered fat. Generally, the results obtained when oral fat was given were comparable to those observed when the same amount of supplementary fat was administered intravenously.

These studies constitute further evidence that suitably prepared intravenous fat emulsions administered to human subjects are utilized for calories and can decrease nitrogen and potassium deficits induced by diets inadequate in caloric content.

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OBJECTIVE EVALUATION OF PATIENTS WITH RHEUMATIC DISEASES

I. Comparison of Serum Glycoprotein, Cold Hemagglutination, C-Reactive Protein, and Other Tests With Clinical Evaluation

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SERUM glycoprotein and seromucoid concentrations have been suggested as objective means for assessing the activity of the rheumatoid process.[1] In a recent study,[2] C-reactive protein was found to be significantly correlated with serum glycoprotein and seromucoid in patients with rheumatoid arthritis.

Hemagglutination tests have been found of value for the diagnosis of rheumatoid arthritis by a number of investigators.[3] Ziff and associates[4] have reported that the test carried out on the fractionated euglobulin fraction of serum protein provides greater specificity and sensitivity. The present study is concerned with comparison of serum glycoprotein and C-reactive protein concentrations with the hemagglutination test as modified by Ziff and co-workers. Antistreptolysin O (ASO) titer, sedimentation rate, and serum γ-globulin were also determined for many of these same patients.

METHODS

Serum glycoprotein (nonglucosamine) was determined by the method of Shetlar and associates.[5] Results of serum glycoprotein estimations are expressed as the ratio of bound carbohydrate to protein (PR*). Total serum protein was determined by the biuret method of Weichselbaum[6] and γ-globulin by the method of Jager and Nickerson.[7] C-reactive protein (CRP) determinations were performed by the capillary precipitant test of Anderson and McCarty,[8] employing commercial C-reactive protein antiserum.† The hemagglutination test was carried out on the serum euglobulin fraction as recently described by Ziff and co-workers.[4] ASO determinations were performed by the method of Ranty and Randall.[10] The erythrocyte sedimentation rate was determined by the Wintrobe method.

Patients with rheumatoid arthritis were classified according to the criteria of Steinbrocker and associates,[9] in addition to which a rating of the current clinical activity was recorded at each visit of the patient. Systemic manifestations including fever, morning stiffness, loss of weight, fatigability, and malaise were appraised along with local manifestations of joint inflammation. These clinical observations were expressed on a four-point activity scale as summarized in Table I.

Normal controls were comprised of laboratory and hospital personnel.

RESULTS

Data resulting from the hemagglutination, C-reactive protein, antistreptolysin, and glycoprotein studies are summarized in Table II.

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*Grams polysaccharide per 100 Gm. of protein.

†Furnished through the courtesy of the Schiefflin Company.

TABLE I. ESTIMATION OF INFLAMMATORY ACTIVITY IN PATIENTS WITH RHEUMATOID ARTHRITIS

CLINICAL ACTIVITY	MORNING STIFFNESS	SLEEP PATTERN	MALAISE, FATIGABILITY, AND WEAKNESS	ACUTE JOINT MANIFESTATIONS	FEVER	APPETITE	PHYSICAL ACCOMPLISHMENTS
I (None)	None	Undisturbed	Absent	Absent	None	Good	Limited only by deformity
II (Mild)	10 min.	Undisturbed	Slight	Transient joint pains	None	Untroubled	Limited only by fatigability and deformity
III (Moderate)	< 30 min.	Mild nocturnal restlessness; early morning awakening	Moderate	Joint swelling without redness or heat	None or slight temp. elevation	Capricious	Limited by joint pain, fatigability, and deformity
IV (Severe)	> 30 min.	Frequent awakening (fatigue)	Pronounced	Joint swelling with redness and heat	Elevation of body temp.	Anorexia	Limited by general malaise, joint pain, fatigability, and deformity

Hemagglutination Titer.—Seventy-seven per cent of the patients with active rheumatoid arthritis and 10 of the 11 inactive cases demonstrated elevated hemagglutination titers. All of the 8 cases of juvenile rheumatoid arthritis and each of the 3 cases of rheumatoid arthritis with psoriasis exhibited positive titers; 6 out of 9 cases of rheumatoid spondylitis (without peripheral joint involvement) were positive. Several cases of rheumatic fever (6 out of 7), periarteritis nodosa (2 of 4), and scleroderma (2 of 4) also had high hemagglutination titers. Several diseases unrelated to the arthritides, i.e., diabetes mellitus, bronchial asthma, carcinoma, and agammaglobulinemia, were investigated and found to exhibit normal hemagglutination titers. Sixteen per cent of normal persons were found to show a weakly positive titer (above 1:14).

TABLE II. SUMMARY OF HEMAGGLUTINATION TITER, ANTISTREPTOLYSIN O TITER, C-REACTIVE PROTEIN, AND GLYCOPROTEIN IN ARTHRITIS PATIENTS

	NO. OF PATIENTS	HEMAGGLUTINATION TITER			CRP (NO. POSITIVE)	ASO (NO. POSITIVE)	GLYCOPROTEIN			
		NO. POSITIVE	AVG.	±S.E.*			MG. %	S.E.	PR	S.E.
Normal	24	4	1:16	± 3	0	0	121	± 4	1.64	±.02
Rheumatoid arthritis, active	44	42	1:83	±18	42	6	187	± 8	2.50	±.06
Rheumatoid arthritis, inactive	11	10	1:54	±10	5	3	156	± 6	2.03	±.08
Rheumatoid arthritis, juvenile	8	8	1:189	±60	4	2	177	± 4	2.24	±.13
Rheumatoid arthritis, psoriatic	3	3	1:233		3	0	202		2.58	
Rheumatoid spondylitis	9	6	1:45	±11	8	1	163	±12	2.10	±.12
Disseminated lupus erythematosus	7	6	1:68	±30	6	1	184	±13	2.62	±.10
Active rheumatic fever	7	6	1:102	±34	4	6	188	±12	2.51	±.11
Periarteritis nodosa	4	2	1:47		3	1	181		2.72	
Scleroderma	3	2	1:149		2	1	150		2.00	
Diabetes mellitus	2	0	1:10		1		123		1.79	
Bronchial asthma	2	0	1:14		1					
Active tuberculosis	3	0	1:12		2	0	238		2.99	
Carcinoma	2	0	1:10		1		170		2.92	
Agammaglobulinemia	1	0	1:2		1		134		2.16	

*S.E.: Standard error of the mean.

C-Reactive Protein.—As previously reported,[2] significant levels of C-reactive protein were found in most cases of rheumatoid arthritis with current activity but seldom in those cases showing no activity. Those cases (5 out of 11) which were rated clinically inactive but who gave positive CRP tests also exhibited elevated glycoprotein levels confirming the correlation between these two tests. As previously reported, elevated CRP occurred in patients with tuberculosis, carcinoma, and agammaglobulinemia.

Antistreptolysin O Titer.—An increased incidence of high ASO titers was found in the general group of rheumatic diseases in contrast to consistently negative ASO titers in the normal individuals as well as the patients with nonrheumatic diseases. This high incidence of positive ASO titers in patients with acute rheumatic fever has been common experience.[11]

Glycoprotein.—Glycoprotein studies of patients with various arthritides, as well as certain other disease states, have been reported previously.[1, 2, 12, 13] Data found in this study are confirmatory of the earlier data.

Sedimentation Rate.—The sedimentation rates were generally high in the patients with active rheumatoid arthritis, only 2 out of 44 not being elevated; however, this phenomenon was found to occur in many inactive as well as active cases.

Gamma Globulin.—Gamma globulin was found to be elevated in most rheumatoid patients, both active (36 of 44) and inactive (9 of 11). Further studies of serum protein changes in these patients will be presented in a later publication.

Correlation of Various Tests With Clinical Activity.—A comparison of the various laboratory data found in active and inactive diseases of connective tissue is presented graphically in Fig. 1. Correlation coefficients of the various

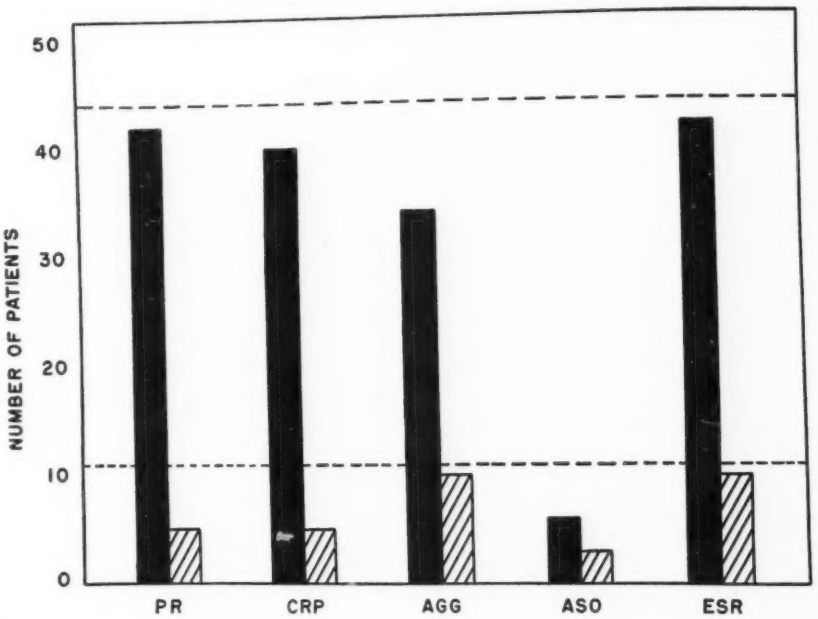

Fig. 1.—Comparison of laboratory data for clinically active and inactive rheumatoid arthritis patients. Forty-four patients rated clinically active (black bars) and eleven rated clinically inactive (crosshatched bars) are presented graphically. The bargraph indicates the number of patients in each group in whom the results exceeded the highest normal value for each test. PR refers to serum glycoprotein expressed as grams of hexose per 100 grams of protein, CRP to C-reactive protein, AGG to the hemagglutination test, ASO to antistreptolysin titer, and ESR to the erythrocyte sedimentation rate.

tests with clinical activity were determined and tabulated (Table III). A highly significant correlation of clinical activity was found with PR and with C-reactive protein. The erythrocyte sedimentation rate was found to be slightly correlated with clinical activity, the correlation coefficient being barely significant at the 5 per cent level. No significant correlations with clinical activity were found with either ASO titer or γ-globulin.

Correlation of Various Tests With Each Other.—PR and C-reactive protein have been reported to show excellent correlation with each other in pa-

tients with rheumatoid arthritis[2]; PR and sedimentation rates were found not to be significantly correlated in such patients.[12] Data obtained in this study confirm the earlier observations. Since the hemagglutination tests as determined in this study were conducted on the euglobulin fraction of the serum protein, some correlation with the serum γ-globulin content might be expected. This was, however, not the case, as shown in Table III; the correlation coefficient for these two tests actually being slightly negative. The correlation coefficient between hemagglutination titer and glycoprotein was not significant.

TABLE III. CORRELATION COEFFICIENTS OF CLINICAL ACTIVITY WITH VARIOUS LABORATORY TESTS IN RHEUMATOID ARTHRITIS PATIENTS

CORRELATION		r	t	PROB.
Hemagglutination titer	X Clinical activity	0.191	1.33	0.2
Antistreptolysin O	X Clinical activity	-0.194	1.19	0.3
γ-Globulin	X Clinical activity	0.135	0.76	0.5
Glycoprotein (PR)	X Clinical activity	0.798	8.67	0.01
C-reactive protein	X Clinical activity	0.515	3.98	0.01
Sedimentation rate	X Clinical activity	0.332	2.17	0.05
Hemagglutination titer	X Glycoprotein (PR)	0.196	1.56	0.2
Hemagglutination titer	X γ-Globulin	-0.051	0.29	0.8
Glycoprotein (PR)	X Sedimentation rate	0.285	1.83	0.1
Glycoprotein (PR)	X C-reactive protein	0.541	4.26	0.01

DISCUSSION

The data presented here on the hemagglutination test is in general agreement with that of Ziff and associates in that the test is positive in most patients with rheumatoid arthritis. However, the data differ from that of Ziff and co-workers in that more positives were found in patients with juvenile rheumatoid arthritis, rheumatoid spondylitis, and rheumatoid arthritis with psoriasis. Comparison of the data of the two studies is summarized in Table IV.

TABLE IV. COMPARISON OF POSITIVE HEMAGGLUTINATION TESTS IN ARTHRITIS PATIENTS

	NUMBER OF POSITIVES*	
	ZIFF AND CO-WORKERS	SHELTAR AND CO-WORKERS
Active rheumatoid arthritis	92%	80%
Inactive rheumatoid arthritis	6/12	8/8
Rheumatoid arthritis with psoriasis	0/6	3/3
Rheumatoid spondylitis	0/11	6/9

*First figure refers to number of positives, second figure to number tested.

The hemagglutination test shows no significant correlation with the acute phase reactant tests including glycoprotein, C-reactive protein, and sedimentation rate, or with clinical activity of the rheumatic process. The value of the test insofar as distinguishing between the various connective tissue diseases would seem to be lessened by our high incidence of positive titers in disseminated lupus erythematosus, scleroderma, rheumatoid spondylitis, and psoriatic arthritis, as well as in rheumatoid arthritis. It would appear, however, that the test serves a useful function in the diagnostic appraisal of patients with rheumatic diseases.

The serum glycoprotein concentration and certain other (acute phase reactant) tests which are less specific for the rheumatic diseases serve as indicators of clinical activity. The PR ratio provides an excellent adjunct to the critical evaluation of these patients under therapy and may be determined with considerable precision. C-reactive protein concentrations, although not as well correlated with clinical activity as PR, are more easily determined and thus may be more readily adapted to the routine laboratory.

The erythrocyte sedimentation rate, though familiar and easily performed, shows comparatively poor quantitative relationship to the degree of clinical activity of the rheumatic diseases. However, as it is not significantly correlated with PR or CRP, it is possible that it expresses some phase of the disease not common to the latter determinations.

Serum gamma globulin concentrations show even less quantitative relationship to activity of the rheumatic diseases than do sedimentation rates, as well as poor correlation to any of the other tests used. This is in contrast to the highly significant negative correlation of glycoprotein and serum albumin.[14]

A high ASO titer usually occurs following infection with Group A hemolytic Streptococci and is generally considered specific for acute rheumatic fever among the rheumatic diseases. The occasional appearance of positive titers among patients with rheumatoid arthritis has been considered either a normal finding or strong evidence for the diagnosis of acute rheumatic fever.[10] Our data would tend to indicate that the ASO titer is elevated somewhat more frequently in patients with the rheumatic diseases (excluding rheumatic fever) than in the normal subjects, though there is no correlation with duration of disease or clinical activity.

Six of the seven patients with acute rheumatic fever showed the rising serial titers of ASO which are characteristic of this disease.

SUMMARY

Serum glycoprotein, C-reactive protein, cold hemagglutination, antistreptolysin O, erythrocyte sedimentation rate, and γ-globulin levels were compared as methods for the evaluation of patients with rheumatic diseases. Serum glycoprotein and C-reactive protein were significantly correlated with clinical activity. A correlation barely significant at the 5 per cent level was found between sedimentation rate and activity. Neither glycoprotein nor C-reactive protein was significantly correlated with sedimentation rate. Hemagglutination titer had no relationship to rheumatoid activity but was usually elevated in patients with rheumatoid arthritis. Serum γ-globulin was generally elevated in patients with rheumatoid arthritis but was not correlated with activity of the disease.

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SOME EFFECTS OF INTRA-ARTICULAR INJECTION OF HYDROCORTISONE ACETATE ON SYNOVIAL FLUID IN RHEUMATOID ARTHRITIS

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STUDIES of normal and arthritic synovial fluid have revealed increased concentrations of sodium in active, untreated rheumatoid arthritis.[1] The striking relief of local joint symptoms in rheumatoid arthritis afforded by intra-articular injection of hydrocortisone[2-4] has prompted us to study possible changes in sodium and potassium concentrations which might be correlated with such periods of clinical improvement.

Prior work correlating increases in protein content[5] and decreases in pH[6] with the severity of joint inflammation and the observations that synovial fluid viscosity increases following intra-articular hydrocortisone[4, 7, 8] made it of additional interest to include observations of protein, pH, and viscosity in our study.

MATERIAL AND METHODS

In the course of routine intra-articular hydrocortisone acetate therapy in a series of more than 500 injections using ambulatory patients, a group was available for study consisting of 34 individuals with active rheumatoid arthritis with involvement of the knee joints. A satisfactory follow-up was successfully obtained in all subjects. Some of these patients were receiving general therapeutic measures (i.e., salicylates, heat, etc.), and two were receiving cortisone orally with some relief of general symptoms, but without alleviation of the symptoms in the joint under study. After aspiration, each joint was injected with 1 to 2 ml. (25 to 50 mg.) of hydrocortisone acetate.† The joint cavity was respirated during the period of clinical improvement, the usual time interval being 5 to 7 days, but ranging from 24 hours to 4 weeks.

The total protein was determined with use of the biuret reagent and crystalline bovine serum albumin as a standard. Sodium and potassium concentrations were determined by means of the Beckman Model DU flame spectrophotometer, with use of a previously described technique.[1] Preliminary electrophoretic studies indicated that true albumin, as determined by salting out with 28 per cent sodium sulfite, could not be accurately determined in synovial fluid.[9]

Immediate determinations of pH values were made in some of the specimens with the Beckman Model G pH meter, with use of the standard Beckman micro bloodtype electrode assembly which allows manipulation of fluids without exposure to air.

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†Hydrocortisone acetate, Merck and Co., Rahway, N. J.

In 12 subjects, simple measurements of viscosity were made before and after treatment using pipette viscosimeters of identical volume and bore diameter. Determinations were made at 37 ± 0.5° C. and compared with standard solutions of glycerol and water in order to express values in terms of "relative viscosity."

RESULTS

The results obtained are shown in Table I. The sodium concentration averaged 142.1 meq. per liter in active rheumatoids before injection of hydrocortisone acetate, as compared to 137.5 meq. per liter after therapy, with an average decrease of 4.6 meq. per liter and a range of the average decrease of 0.2 to 29.0 meq. per liter. No correlations with time interval after injection were obtained. The individual differences in the values obtained before and following hydrocortisone acetate therapy were evaluated by applying calculations for Student's "t" distribution.[10] The probability of these changes being due to chance is less than 1 per cent. The average potassium concentration before and after hydrocortisone acetate injection remained at 4.6.

TABLE I. SYNOVIAL FLUID IN RHEUMATOID ARTHRITIS BEFORE AND AFTER HYDROCORTISONE ACETATE INJECTION

	NUMBER PATIENTS	BEFORE (MEAN)	STANDARD DEVIATION	AFTER (MEAN)	STANDARD DEVIATION	STUDENT'S "t"
Sodium (meq./L.)	34	142.1	7.6	137.5	8.3	3.14*
Potassium (meq./L.)	34	4.6		4.6		
Total protein (Gm./100 ml.)	34	5.3	1.2	5.2	1.1	
pH	6	7.31	0.10	7.44	0.04	0.34†
"Relative viscosity"	12	34.3	29.4	85.7	78.3	2.52‡

*Significant at $p < 0.01$.
†Not significant at $p > 0.05$.
‡Probably significant at a slightly > 0.01.

The average total protein values of the synovial fluid before and after therapy were 5.3 and 5.2 Gm. per cent, respectively.

The average initial pH value found in six patients with rheumatoid arthritis was 7.31; five fluids were within the range of 7.17 and 7.36, and one was 7.50. After the intra-articular injection of hydrocortisone acetate the average pH value was 7.44. All these fluids were within the range of 7.4 and 7.5, and only one patient failed to show an increase in pH. Another patient, from whom three periodic samples were obtained, showed an initial fall in pH associated with a failure of clinical response followed by both clinical improvement and an increase in pH after a second injection. However, analysis using Student's "t" revealed the differences to be without significance.

In the subjects studied, the "relative viscosity" rose following treatment from an average value of 34.3 to 85.7 with an average increase of 51.4, the range

being from –6 to +213. These differences, as analyzed with use of Student's "t,"[10] had a probability of being due to chance of slightly greater than 1 per cent.

DISCUSSION

Before hydrocortisone acetate therapy, the average sodium concentration of rheumatoid arthritic synovial fluid (142.1 meq. per liter) was close to that in normal blood serum[11] (144.7 ± 3.81 meq. per liter) and rheumatoid blood serum[12] (144.1 ± 3.67 meq. per liter). After intra-articular injection of hydrocortisone acetate, the average sodium ion concentration in rheumatoid synovial fluid (137.5 meq. per liter) closely approached that obtained[1] for normal synovial fluid (136.1 meq. per liter). These changes are consistent with the concept that rheumatoid arthritis is associated with a change in permeability of the joint tissues and a shift of the synovial fluid ionic composition toward that of the blood plasma. The particle size of hyaluronic acid would also seem to play a role in determining the sodium concentration. The injection of hyaluronidase into the synovial fluid of dogs caused a marked increase in the sodium concentration in vivo.[13] There is an apparent decrease in the particle size of hyaluronic acid in synovial fluid in rheumatoid arthritis,[14, 15] and intra-articular injection of hydrocortone has been associated with increases in synovial fluid viscosity and degree of polymerization of the hyaluronic acid.[4, 7, 8] However, the essentially unchanged value for proteins in relation to the improvements produced by hydrocortisone acetate are not consistent with the correlation previously reported[5] between protein content and the severity of the disease. The abnormally elevated values for proteins noted after treatment were probably in keeping with the transient nature of improvement observed following intra-articular hydrocortisone acetate.

SUMMARY

The average sodium ion concentration, originally close to the serum values, decreased significantly toward the value for normal synovial fluids after intra-articular injection of hydrocortisone acetate in 34 patients with rheumatoid arthritis. The average protein and potassium concentrations remained unchanged following injection. Studies of pH were insufficient in number from which to draw definite conclusions. Viscosity measurements on 12 subjects revealed significant increases after treatment.

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IMMUNOTHROMBOCYTOPENIC PURPURA DUE TO QUINIDINE

Report of Four New Cases With Special Observations on Patch Testing

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IT IS well known that a number of drugs, including quinidine, may be responsible for thrombocytopenic purpura. A review of the literature reveals over twenty cases of quinidine-induced thrombocytopenic purpura, most of which are noted in the combined bibliographies of two recent papers.[1, 2] That this disease is by no means as rare as has been frequently asserted in the literature is attested to by the fact that it has been possible for us to detect and study four new cases at one hospital in a period of seven months.

The purpose of the present communication is to report the clinical features of these four cases of drug-induced thrombocytopenic purpura and to present data on in vitro and skin testing techniques.

CASE REPORTS

Case 1 (No. 66465).*—This 69-year-old white man was hospitalized because of a recurrent supraventricular tachycardia for which a few brief courses of quinidine sulfate had been given over the previous two years. The patient was digitalized and started on quinidine sulfate on the day of admission (Oct. 31, 1954). Sinus rhythm was restored and the medications were continued until epistaxis and a purpuric eruption appeared on the third hospital day after a total dose of 5.8 Gm. of quinidine sulfate. At this time the hemoglobin was 10.5 Gm. per cent, the white blood cell count normal with a marked shift to the left, and the platelets virtually absent on stained blood films. There was no eosinophilia. Rumpel-Leede's test was strongly positive, clot retraction was absent in 24 hours, and the bleeding time was greater than 35 minutes. The clotting time was normal. A sternal bone marrow examination on the same day revealed an increase in the number of megakaryocytes and virtual absence of platelet production; increased erythropoietic activity was also present. A reticulocytosis of 12 per cent developed by the eighth hospital day in association with a fall in hemoglobin to 8.5 Gm. per cent.

The quinidine sulfate was promptly discontinued and ACTH (40 units gel per day) was administered for the next four days. The purpura disappeared on the sixth hospital day and the platelet count returned to normal by the eighth hospital day.

Special studies of the patient's serum proved the existence of a hemolysin and a thromboagglutinin which were active only in the presence of quinidine. Detailed data relating to the hemolysin have been presented elsewhere.[3] It was also possible to demonstrate and study (vide infra) the localized development of capillary sensitivity and damage by means of a cutaneous patch test in this patient.

Case 2 (No. 71528).—On March 12, 1955, a 54-year-old white woman was transferred to Montefiore Hospital for splenectomy with a diagnosis of "chronic idiopathic thrombocytopenic purpura."

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*This case has previously been reported in greater detail.[3]

Careful history revealed that the patient had taken quinidine sulfate on several occasions since July, 1954, because of "rapid heart action." In January, 1955, she developed cutaneous purpura and bleeding "from the mouth and throat" for which she received transfusions, vitamins C and K, cortisone, and ACTH gel, despite which the bleeding manifestations intermittently reappeared. Quinidine sulfate had been taken intermittently during this period.

Physical examination on admission revealed cutaneous purpura and ecchymoses of the conjunctivae and palate. The spleen and lymph nodes were not enlarged.

Rumpel-Leede's test was moderately positive on admission. On the next day, the hemoglobin measured 13 Gm. per cent, the leukocytes 11,700 per cubic millimeter with a slight shift to the left, and the platelets 79,000 per cubic millimeter. Clot retraction was slightly depressed, bleeding time was 9 minutes, and clotting time was normal. Eosinophilia, reticulocytosis, erythrophagocytosis, and L.E. cells were absent in suitable preparations. Coombs tests were negative.

Bone marrow films (some received from the previous hospital) revealed: In January (during period of quinidine ingestion and thrombocytopenic purpura), the number of megakaryocytes was markedly increased and platelet production was absent; in February (during period free of quinidine ingestion and when platelet count was normal), the number of megakaryocytes was still increased but platelet production was normal; on March 16 (5 days after the present admission and at least 6 days after the last dose of quinidine), during clinical remission, the megakaryocytes were essentially normal in number and productivity.

Quinidine was strictly withheld from the patient from the time of admission to Montefiore Hospital. Cortisone therapy was gradually discontinued. All evidence of abnormal bleeding disappeared within 48 hours of admission as the platelet count rapidly returned to normal.

On the day of admission the diagnosis of quinidine-induced thrombocytopenic purpura was confirmed by the demonstration of complete inhibition of clot retraction in vitro by the addition of quinidine sulfate to the patient's whole blood. The patch tests were negative. Platelet agglutinins were demonstrated in the patient's serum.

Case 3 (No. 69394).—Because of ventricular premature contractions, this 70-year-old woman had been given 0.6 Gm. of quinidine sulfate daily for the seven months prior to admission to Montefiore Hospital on April 30, 1955. She was admitted because of the appearance of a purpuric eruption.

Physical examination revealed generalized purpura of skin and mucous membranes. Lymphadenopathy and splenomegaly were absent.

On the day of admission the peripheral blood showed marked thrombocytopenia, but three days later the platelet count was 97,500 per cubic millimeter and thereafter was in the normal range. Rumpel-Leede's test was positive on admission. By the seventh hospital day the bleeding and clotting times were within normal limits. Blood hemoglobin level and leukocyte count and differential were normal. The Coombs test was negative and there was no laboratory evidence of hemolysis or the L.E. phenomenon. Examination of a bone marrow film on the fifth hospital day revealed a slight increase in number of megakaryocytes but normal maturation.

Quinidine was discontinued and the patient was placed on metacortandracin, 20 mg. daily, at the time of admission. All evidence of active bleeding disappeared by the fourth hospital day. On the sixth hospital day it was demonstrated that addition of quinidine solution to the patient's whole blood inhibited clot retraction. Quinidine patch tests were negative.

Case 4 (No. 73438).—Because of cutaneous ecchymoses and petechiae of one day's duration, this 39-year-old white man was admitted to Montefiore Hospital on June 4, 1955. The patient had known rheumatic heart disease and had used quinidine occasionally during the past six months. He completely denied taking quinidine during the month prior to admission but admitted using phenobarbital and aspirin during the previous week.

Physical examination was unremarkable except for the purpura and evidence of aortic stenosis.

Rumpel-Leede's test was strongly positive on admission but was negative three days later. The hemoglobin was 16.5 Gm. per cent and the leukocytes unremarkable except for a mild eosinophilia. An L.E. preparation was negative and heterophile antibodies were absent. On the third hospital day the platelet count was 187,000 per cubic millimeter, the prothrombin, bleeding, and clotting times were normal, and clot retraction was noted to be "poor." Two days later the platelet count was 313,200 per cubic millimeter.

The admission diagnosis was idiopathic thrombocytopenic purpura. The patient was treated with bed rest alone. No evidence of fresh bleeding occurred following admission to the hospital.

When the patient was seen by us on June 8, a careful history revealed that he was not "positive" that he had not taken quinidine by mistake since his pill bottles were kept side by side. Accordingly a quinidine patch test was applied and found to be positive 24 hours later (over 200 petechiae in a 2.5 cm. square) when a tourniquet was applied. Marked inhibition of clot retraction was noted a few days later when quinidine solution was added to the patient's whole blood. Similar tests run with phenobarbital and aspirin were negative.

On June 25, a test dose of 50 mg. quinidine sulfate was administered orally. The platelet count fell from 250,000 to 120,000 in 10 minutes, rose to 150,000 in 25 minutes and returned to control values 90 minutes following the test dose. During this test period, capillary fragility and bleeding time remained normal.

Techniques for Demonstration of Role of Suspected Drug in Production of Thrombocytopenic Purpura.—A variety of procedures are now available to demonstrate the causal relation of a drug to an episode of thrombocytopenic purpura. These are noted below in outline form:

I. *In vivo techniques:*

A. Direct: Production of thrombocytopenia by a test dose or repeat course of suspected drug. Although this is the most straightforward and sensitive of these procedures,[8] the amount of the drug required cannot be estimated accurately and this approach is always potentially dangerous, a number of alarming hemorrhagic reactions having resulted in sensitive individuals.[1, 6, 14]

B. Indirect: Transfusion experiment: production of thrombocytopenia in a normal recipient following transfusion of blood from a patient who has recently experienced an episode of drug-induced immunocytopenia and administration of the drug to the recipient.

II. *In vitro techniques:*

A. Direct: Microscopic demonstration of agglutination and/or lysis of platelets in the presence of patient's serum and the drug.

B. Indirect: Demonstration of alteration of platelet function or behavior in presence of sensitive serum and the drug:

1. Inhibition of clot retraction.
2. Acceleration of optical clearing of platelet-rich plasma.
3. Impairment of prothrombin utilization.

III. *Patch tests:* Demonstration of capillary sensitivity (production of local purpura) by topical application of the drug to the patient's skin.

Methods and Results of In Vitro and Patch Test Techniques in Reported Cases.—

Platelet agglutinins:* The agglutinability of platelets by a serum factor in the presence of quinidine was demonstrated in two cases in which the appropriate test (1) was employed. In Case 1 the results were moderately positive and in Case 2 weakly positive.

The thromboagglutinating and/or thrombolytic effect of a serum factor in the presence of the causative drug has been demonstrated on a number of occasions.[1, 4, 7-9, 11] In the case of idiopathic thrombocytopenic purpura it has been shown[13] that a serum factor exerts similar effects upon platelets without addition of a drug to the system.

Clot-retraction studies: The inhibition of clot retraction (due to the effect on platelets) by the addition of small amounts of the offending drug to fresh whole blood from the patients who were convalescing from purpura due to Sedormid[8] and quinidine[7, 11] has been previously noted. Using silicone techniques, Larson,[11] and Barkham and Tocantins[7] were able to impair clot retraction by adding quinidine sulfate, in final concentrations of 4.5 to 10.0 mg. per cent to sensitive whole blood. Quinine, the optical isomer of quinidine, exerted no such effect. The technique described by Macfarlane[15] for the measurement of clot retraction was the most convenient in our experience. Glass centrifuge tubes, graduated to 15 ml., were employed. Three milliliters of test solution (quinidine and drug controls made up in 0.9 per cent saline) was thoroughly mixed with 6 ml. of the patient's fresh whole blood and readings were made after two hours' incubation at 37.5° C. Per cent inhibition of clot retraction was calculated from the following formula, in which "ml. serum NaCl" represents the volume of serum expressed from the control tube and "ml. serum QD" represents the volume expressed from the tube containing quinidine:

$$\text{per cent inhibition} = \frac{\text{ml. serum NaCl} - \text{ml. serum QD}}{\text{ml. serum NaCl}} \times 100$$

As is shown in Fig. 1, although maximal inhibition of clot retraction was obtained with the highest concentrations of quinidine sulfate employed, significant inhibitions were also obtained with low concentrations.

The addition of quinidine to the blood of 20 normal controls did not influence clot retraction. Likewise, the addition to the patients' bloods of a number of control medications (including quinine, procaine amide, and all drugs with which the patients had recently had contact) produced no effect on clot retraction.

In two of our cases, serial determinations of clot-retraction inhibition were made at various times following the clinical episode of thrombocytopenic

*We are grateful to E. Vorsanger, L. Weisfuse, P. W. Spear, and M. Sass of the Brooklyn Veterans Administration Hospital for performing these platelet-agglutination studies by the technique previously described by them.[1]

purpura. In Case 2 clot retraction was completely inhibited by quinidine during the first five days but was only slightly inhibited 10 days later. In Case 4 the inhibitory effect decreased some 15 per cent in the course of 7 days.

Three tests were performed in which 3 ml. of serum from Cases 2 and 4 and 1.5 ml. quinidine were mixed with 3 ml. of whole blood of three normal subjects. The observed inhibition of clot retraction measured 6.5 per cent, 18 per cent, and 41 per cent.

Patch tests for skin (capillary) sensitivity: To our knowledge, Ackroyd's investigations[14] are the only ones in which localized capillary damage (purpura) could be produced in the skin of patients who had recovered from drug-induced thrombocytopenia. Ackroyd applied a super-saturated solution of Sedormid in propylene glycol (Sedormid being insoluble in water) to the skin of patients convalescing from Sedormid-induced thrombocytopenic purpura and, after 24 to 48 hours' contact, was able to visualize localized true purpura. The purpura was enhanced by application of a capillary stress (positive pressure capillary fragility test). The patch test was positive in only 2 of the 3 patients studied by Ackroyd and was considered less sensitive than the in vitro platelet techniques employed by him.

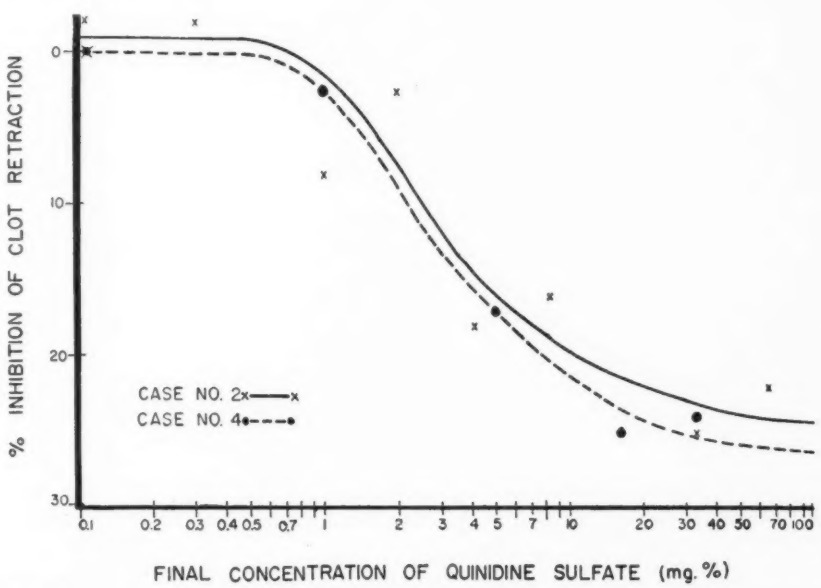
% INHIBITION OF CLOT RETRACTION
0
10
20
30
CASE NO. 2
CASE NO. 4
0.1 0.2 0.3 0.4 0.5 0.7 1 2 3 4 5 7 10 20 30 40 50 70 100
FINAL CONCENTRATION OF QUINIDINE SULFATE (mg.%)

Fig. 1.—Relation of in vitro concentration of quinidine sulfate solution in mg. per cent to degree of inhibition of clot retraction in two cases. (Semi-log plot.) (See text.)

Several investigators have unsuccessfully attempted to demonstrate patch test sensitivity in patients who developed thrombocytopenic purpura secondary to drug ingestion. Bigelow and Desforges[4] used a moistened tablet of quinidine sulfate and left it in contact for only 10 hours. Larson[11] employed a relatively weak solution of quinidine sulfate solution (80 mg. per cent) with an adequate period of contact (24 hours). Bolton and Young[10] do not give the details of their technique of patch testing appropriate individuals with quinine, sulfamezathine, and quinidine.

By our technique, patch tests were positive in two (Cases 1 and 4) of our four patients. A number of special observations pertaining to this reaction were made (Figs. 2 to 5). The patches were made up of gauze to which various strength solutions of quinidine sulfate or quinidine sulfate paste were applied.

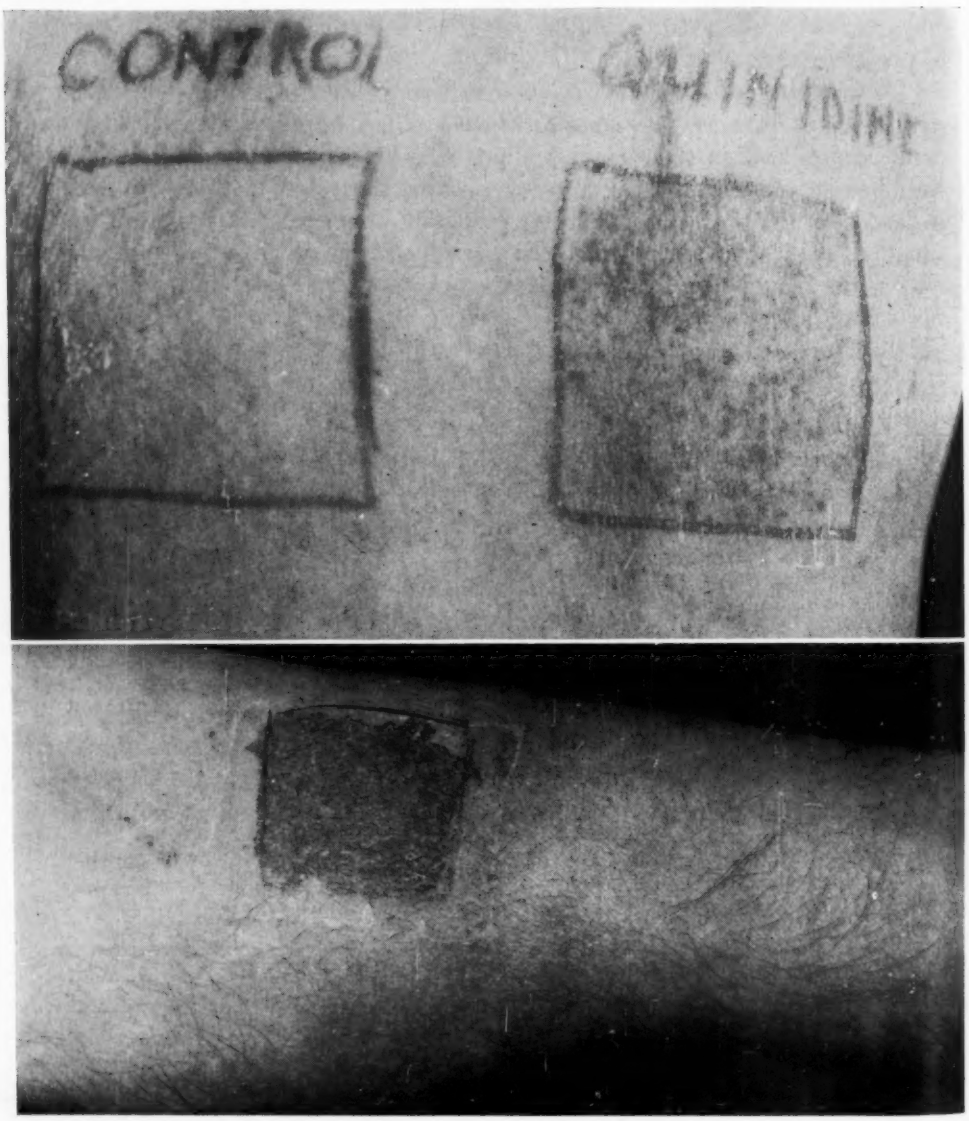

Fig. 2.—(Case 1.) Grading of purpuric reactions following patch testing with quinidine paste: upper left negative (0); upper right, moderate (2+); lower, intense (4+).

The solutions were made up in 0.9 per cent saline or water and the paste was prepared by heating quinidine sulfate powder in a small volume of water. These patches were left in contact with the patients' arms or backs for lengths of time varying from one-half to over 48 hours. Upon removal of each patch, the skin was immediately examined for any reaction, the residual paste or

solution removed, and a positive or negative pressure test of capillary fragility performed (application of blood pressure cuff at mid-point between systolic and diastolic pressure for 10 minutes or suction apparatus). Fig. 2 demonstrates the appearance of the positive skin test to quinidine paste in Case 1. Fig. 2 (lower) shows a reaction of such intensity that it was impossible to innumerate the separate petechiae, while (upper right) shows a moderate reaction in which over 200 petechiae were counted in a 2.5 cm. square area.

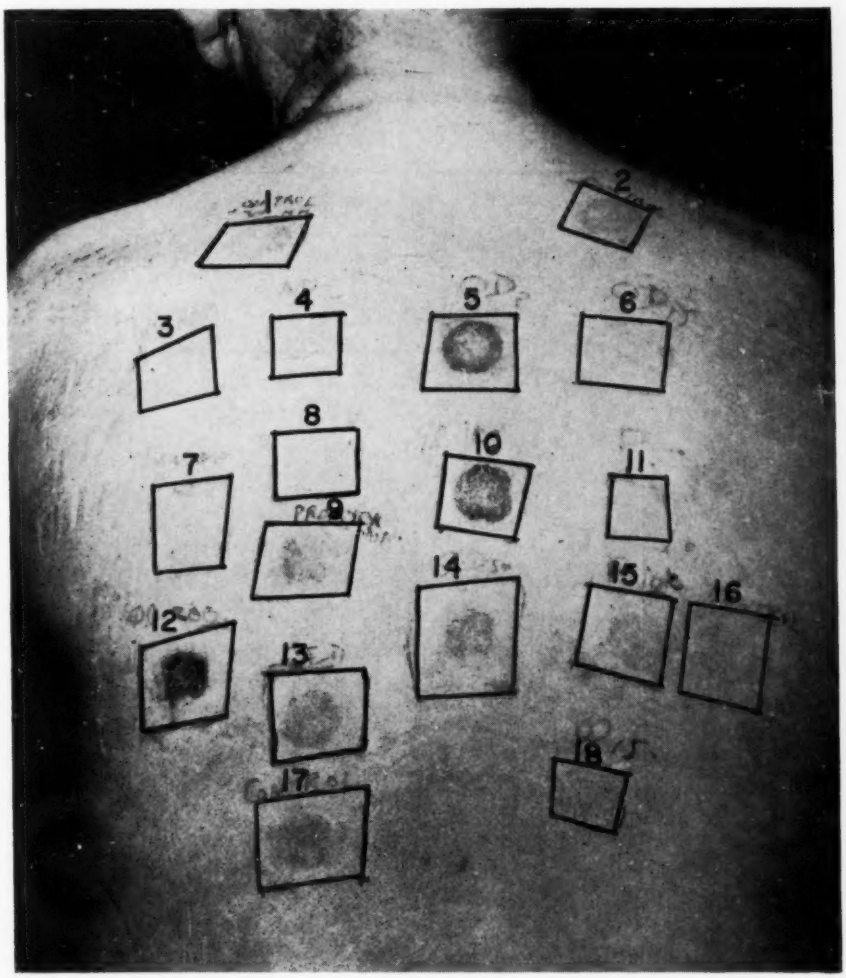

Fig. 3.—Experiment showing reactions obtained in various patch test areas with the use of negative pressure technique. Positive reactions occurred only in areas in contact with quinidine paste (*5*) and 50, 100, and 200 mg. per cent (*14*, *10*, and *12*, respectively) quinidine sulfate solutions. True purpura does not result from contact with 15 mg. per cent quinidine sulfate solution (*6*), quinine sulfate paste (*7*), quinine solution (*18*), phenobarbital paste (*3*), APC paste (*4*), digoxin paste (*8*), Cedilanid (*13*), or procaine amide paste or solutions (*9*, *11*, and *15*). Blank controls are *1*, *2*, *16*, and *17*.

A large number of control drugs and other drugs which the patients were known to have recently ingested produced negative skin tests, as shown in Fig. 3. Quinidine paste produced much stronger reactions than dilute solution of quinidine sulfate (Fig. 4), where the intensity of reaction to quinidine paste

is compared to 200 mg. per cent solution, 100 mg. per cent solution, and 50 mg. per cent solution. Capillary sensitivity to quinidine in Case 1 as revealed by patch tests persisted for more than 140 days following the initial episode of quinidine purpura and withdrawal of the drug (Fig. 4).

The time required to damage sensitive capillaries in the presence of circulating antibody and topical quinidine applied as a paste patch test is plotted in Fig. 5, curve *A*. Patch tests were left in contact with the skin for periods of time varying from one-half to over 48 hours, and immediately upon removal, positive pressure was applied in the form of a tourniquet on the arm. Maximal capillary damage was produced after 24 hours' contact and longer contact produced no further effect. The next step was to leave all patch tests in contact with the skin for the full 24-hour period and apply the

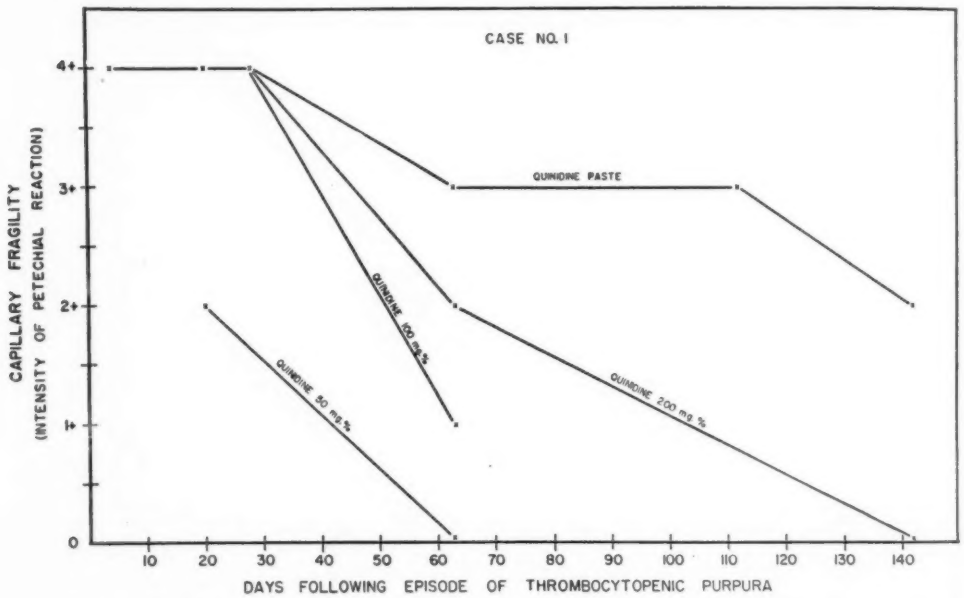

Fig. 4.—Duration and degree of capillary sensitivity to various strengths of quinidine paste and solution. Note that significant sensitivity persists for more than 140 days after the clinical episode of purpura and that quinidine paste is a stronger stimulus than the dilutions of quinidine solution employed.

tourniquet at intervals of from one-half to 72 hours after the patch had been removed. The intensity decreased progressively as longer intervals were permitted to elapse between removal of patch and application of positive pressure (Fig. 5, curve *B*). With use of the same patch test techniques with quinidine paste, 15 control patients were tested and showed no reaction. Several of these patients had received quinidine in the past with no purpura. Topical application of cortisone to the skin test area did not diminish the intensity of the positive reaction in the two patients with reactions to quinidine paste. Circulating platelets remained normal throughout the skin-test period, and no systemic effect was observed from quinidine applied to the skin.

Passive transfer: Serum from the patients who demonstrated the most positive skin tests (and presumably had the highest titer of circulating anti-

body) was injected intradermally into a control subject and challenged immediately, one-half hour, and 24 hours later with an intradermal injection of solution of quinidine and positive pressure in an attempt to demonstrate passive transfer (Prausnitz-Kustner reaction). As in the case of Ackroyd working with Sedormid,[14] we were unable to demonstrate passive transfer of the antibody.

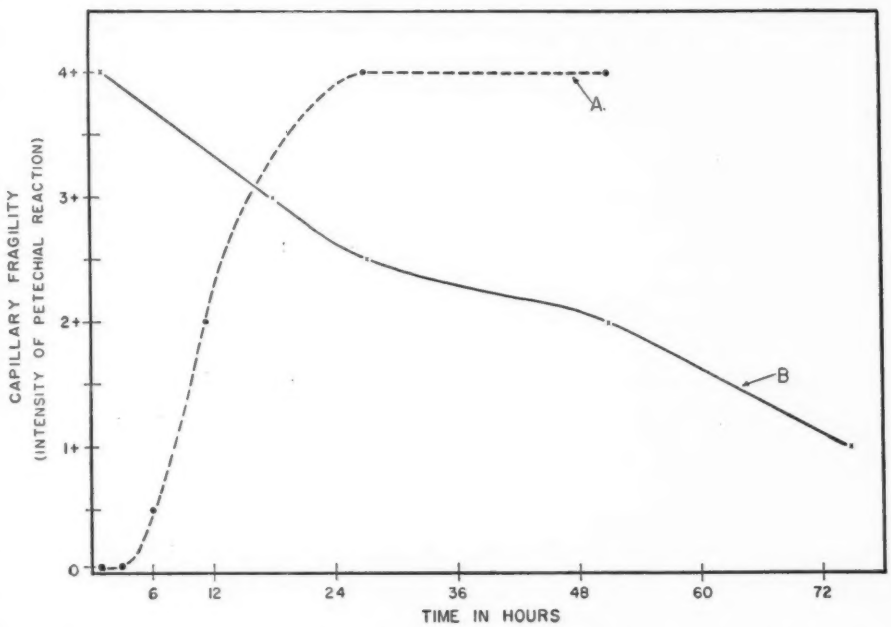

Fig. 5.—Temporal relations of quinidine application (patch test) to capillary fragility. (Case 1.) Curve *A*, quinidine paste patch tests left in contact with the skin for varying periods of time from one-half hour to over 48 hours. Positive pressure applied immediately upon removal of patch and capillary fragility estimated 0 to 4+. Note that essentially maximal capillary damage was produced by 24 hours' contact and that significant damage was produced by 6 to 12 hours' contact.

Curve *B*, quinidine paste patch tests left in contact with skin for 24 hours. Positive pressure applied at varying intervals of one-half hour to more than 72 hours after removal of the patches and capillary fragility estimated. Note that capillary integrity is restored in a fairly linear manner.

In Case 4, virtually identical relationships were obtained.

DISCUSSION

The immunologic nature of *idiopathic* acquired hemolytic anemia,[16] idiopathic thrombocytopenic purpura,[12, 13] and idiopathic leukopenia[17-19] has been adequately established in a large number of cases. In recent years the immunologic mechanism of a number of *drug-induced* cytopenias has also been demonstrated (Table I). It has been shown that a serum factor from a sensitized patient is capable of agglutinating and/or lysing the appropriate blood cells. Unlike the idiopathic immunocytopenias, however, this effect requires the presence of minute quantities of the etiologic drug as well as the serum factor, blood cell, and complement. As is characteristic of other known immunologic reactions, agglutination occurs in the absence of complement, but complement

is required for the production of lysis. When studied the cytopenic factors have been shown to behave physically and chemically like antibodies, and in a few cases,[3, 20, 21] complement fixation has been conclusively demonstrated.

Table I lists cases in which evidence clearly supporting the existence of an immunologic mechanism in drug-induced cytopenias has been obtained. It is to be noted that such evidence is available for hemolytic anemia and leukopenia as well as for thrombocytopenia due to drugs.

TABLE I. MAJOR POSITIVE DATA IN DRUG-INDUCED IMMUNOCYTOPENIAS

DISEASE	ETIOLOGIC AGENT	REFERENCE	NO. OF CASES	REACTIONS DEMONSTRATED				
				AGGLUTINATION	LYSIS	COMPLEMENT FIXATION	PATCH TEST	INHIBITION OF CLOT RETRACTION
A. Thrombocytopenia purpura	Quinine	Grandjean[9]	1		+			
	Quinine	Bolton and Young[10]	1	+	+			
	Sedormid	Ackroyd[8, 14, 20, 21]	3	+	+	+	+	+
	Sulfamethazine	Bolton and Young[10]	1	+	+			
	Quinidine	Bigelow and Desforges[4]	2	+	+			
	Quinidine	Larson[11]	1	+				+
	Quinidine	Bolton and Young[10]	1	+				
	Quinidine	Barkham and Tocantins[7]	1	+	+			
	Quinidine	Weisfuse, Spear, and Sass[1]	1	+				
	Quinidine	Present authors	4	+			+	+
B. Hemolytic anemia	Fuadin	Harris[22]	1	+				
	Quinidine	Freedman, Barr and Brody[3]	1	+	+	+		
C. Leukopenia	Sulfapyridine	Moeschlin[17]	1	+				

This table includes cases in which the drug was required to be present in order to activate the experimental system. Although it seems likely that pyramidon or one of its active metabolites is needed to produce leukocyte effects in pyramidon-induced leukopenia,[25, 26] this point has not yet been settled.

In view of the above, it is not surprising that idiopathic and drug-induced thrombocytopenic purpuras have striking clinical similarities. When the bone marrow is aspirated during the active course of quinidine-purpura, at which time a significant level of the drug presumably exists in the blood and tissues, it is indistinguishable from that of the idiopathic disease. Bone marrow examinations have been performed in 10 previously reported cases of quinidine-induced thrombocytopenic purpura. In many cases the megakaryocytes are described as normal in number and morphology. Occasionally[1, 4-7] a reduction

in number and/or productivity of these cells has been noted. According to Ackroyd's review[8] similar observations have been made in thrombocytopenia due to Sedormid. Unfortunately it is rarely stated when, in relation to the clinical episode of purpura, the bone marrow was aspirated.

In one of the present cases, a bone marrow examination was not performed. In a second case, the marrow was essentially normal when examined following recovery from the thrombocytopenia. In the remaining cases (Cases 1 and 2) bone marrow smears were indistinguishable from those of idiopathic thrombocytopenic purpura. In these cases, the aspirate was taken during the clinical episode of thrombocytopenic purpura.

Peripheral eosinophilia is occasionally observed in both the idiopathic and drug-induced disorders, and significant lymphadenopathy and splenomegaly are usually absent. Further evidence for a common immune mechanism in idiopathic and drug-induced thrombocytopenias is provided by occasional observations of dual or pancytopenias in both types of conditions.[3, 17, 23]

The available evidence indicates incontrovertibly that a factor with the characteristics of an antibody is often present in the serum of patients with drug-induced cytopenias. The fact that the causative drug must be present to produce the cellular alterations leads to the concept that the drug acts as a hapten.[3, 8] The link of hapten to cell in these reactions, however, has been shown to be moderately[21] or highly[3] unstable. Accordingly it has been postulated[3, 21] that the infrequency of occurrence of drug-induced cytopenias may be related to the laxity of union of the hapten-cell antigen.

Why only certain individuals develop immunocytopenias due to drugs remains unanswered. We are inclined to believe, as others[16, 24] have suggested, that for some obscure reason the reticuloendothelial system of certain individuals hyperreacts to certain ordinarily weak antigenic stimuli.

Although amenable to other interpretation, we believe that Ackroyd's hypothesis of the antigenic similarity or identity of platelet-protein, megakaryocyte-protein, and capillary endothelium-protein is the most reasonable explanation of the observations on platelets, bone marrow, and patch test in drug-induced thrombocytopenia. According to this interpretation a single antibody could react with any of these cells in the presence of the hapten (drug). We believe that the occurrence of localized purpura in patch testing is independent of platelet phenomena and is due to damage to the capillary endothelial wall in the presence of local quinidine and through the fixation of circulating antibody.

SUMMARY

Four cases of quinidine-induced immunothrombocytopenic purpura, diagnosed at one hospital within a period of seven months, are reported. A high "index of suspicion" is necessary to differentiate drug-induced from primary thrombocytopenic purpura. The essence of treatment is immediate discontinuation of the causative drug.

Drug-induced cytopenias are frequently mediated by immunologic mechanisms. The diagnosis of quinidine-induced thrombocytopenias can be estab-

lished by a number of special techniques which are described. The most convenient screening test is based upon inhibition of clot retraction of the patient's blood in the presence of quinidine.

Patch tests produced localized purpuric reactions in two of our four patients. Analysis of data obtained from these patch tests and from bone marrow examinations suggests that platelets, megakaryocytes, and capillary endothelial cells have a common antigen which is implicated in the immunothrombocytopenias.

We are indebted to Dr. Louis Leiter, Chief of the Division of Medicine, Montefiore Hospital, for his continual encouragement and constructive criticism of this project.

Gratitude is due Drs. Alter Weiss, Ira Rubin, and Ephraim Katz for allowing us to study, respectively, Cases 1, 3, and 4. We owe many thanks to A. C. and I. S., two of the patients in the series, for their cooperation in carrying out these studies.

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ADDENDUM

Since this paper was written, one of us (E. A. B.) has observed two additional cases of quinidine purpura in which patch tests and in vitro clot-retraction inhibition tests were done.

CASE 1.—A 78-year-old white woman with hypertension and a supraventricular tachycardia was treated with quinidine (total of 2.5 Gm.), over a five-day period and developed weakness, petechiae, and rectal bleeding. On admission to the hospital, the platelet count was 30,000 and rose to 198,000 over the next seven days on withdrawal of the quinidine and ACTH therapy. A quinidine patch skin test was positive as was the clot-retraction inhibition test.

CASE 2.—A 76-year-old white woman had received quinidine on several occasions over a five-month period for paroxysmal auricular fibrillation. Purpura had appeared each time several hours after taking of the drug, but five days prior to admission while taking quinidine, she developed bloody stools, petechiae, and bleeding gums as well as purpura. The petechial rash and bleeding faded rapidly on withdrawal of quinidine. The clot-retraction inhibition test was positive, but the patch test was negative.

THE EFFECT OF DICUMAROL ON CERTAIN FACTORS OF BLOOD COAGULATION

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DICUMAROL and other coumarin derivatives have been and are widely used in the treatment and prevention of thrombotic and thromboembolic phenomenon. Such therapy is usually regulated by the Quick prothrombin time determination or some modification of it. It is accepted that the Quick prothrombin time is not always a true reflection of prothrombin concentration, but that it may be influenced by many other factors.[1, 2] Owren[3] and Koller,[4, 5] for example, have stated that the prolonged prothrombin time produced by dicumarol is due in a large part to a decrease in Factor VII. However, to date there is no definite knowledge as to the mode of action of dicumarol in the body other than that it may be counteracted by the administration of vitamin K_1.

To demonstrate how various factors in the coagulation mechanism are affected by dicumarol, the following determinations were done daily on blood from treated patients before, during, and after therapy: free prothrombin time, total prothrombin time, labile factor, thrombin time, and thrombin production time of oxalated plasma; the free prothrombin time and total prothrombin time of plasma to which a small amount of serum was added; and the serum antithrombin time. Nine patients whose ages varied from 57 to 79 were studied. None had clinical or laboratory evidence of liver disease.

METHODS

The free prothrombin time, labile factor, and antithrombin time were determined by the methods previously described.[6]

In 1949, Quick and Stefanini[7] demonstrated that prothrombin exists in both an active and inactive form and that the inactive form becomes active on contact with glass. When all of the prothrombin is activated, the prothrombin time of plasma is shortened from 12 seconds to 8 to 9 seconds. This latter figure is designated as the total prothrombin time. Furthermore, Quick[8] has shown that the administration of dicumarol results in a prolongation of both the total prothrombin time and the free prothrombin time of plasma.

The procedure for determining the total prothrombin time was the same as that recently reported.[9] One milliliter of oxalated plasma was stored in an open glass test tube at 4° C. for 24 to 48 hours. The prothrombin time was then determined in the usual manner after labile factor, which diminishes during storage, was replenished by the addition of deprothrombinized rabbit plasma. One-hundredth milliliter of rabbit plasma, deprothrombinized with tricalcium phosphate, was added to 0.1 ml. of the stored plasma.

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To evaluate the extent to which a dicumarol-induced Factor VII deficiency contributed to the prolongation of the free and total prothrombin times, these determinations were also made on plasma to which human serum (as a source of Factor VII) had been added. One-hundredth milliliter of serum was added to 0.1 ml. of the plasma to be tested. In order to preclude the addition of prothrombin by this procedure, the serum was prepared from the blood of a boy who has a congenital hypoprothrombinemia but a normal amount of Factor VII. This boy is one of the patients recently reported by Quick and his associates.[9] The freshly prepared serum was frozen in small quantities and stored at -20° C. until ready for use.

Thrombin Production Time.—One-tenth milliliter of hemolysate was added to 1.0 ml. of oxalated plasma and the mixture diluted to 10.0 ml. with cold distilled water. In a cold room kept at 4° C., carbon dioxide gas was bubbled through the solution for 10 minutes. The solution together with the precipitate which formed were transferred to a centrifuge tube and centrifuged for 10 minutes at 2,000 r.p.m. After the supernatant fluid was decanted, the tube was inverted and allowed to drain; the sides of the tube were dried with a roll of filter paper. The tube was then removed from the cold room, and the precipitate was redissolved in 0.9 ml. of 0.85 per cent saline. Solution of the precipitate was facilitated by the addition of 1 drop of 5 per cent sodium bicarbonate which raised the pH to approximately 7. One-hundredth milliliter of 0.1 M calcium chloride was added and the mixture placed in a 37° C. water bath. As the strands of fibrin formed, they were removed by wrapping them about a glass stirring rod. A clear solution thus obtained was tested for thrombin content in the following manner: 0.1 ml. of the thrombin solution was blown from a serologic pipette into 0.2 ml. of rabbit plasma deprothrombinized with tricalcium phosphate. The time required for a clot to form was measured with a stop watch. The thrombin production time obtained with preparations in normals and from the patients in this study, prior to treatment, varied from 3 to 6 seconds.

The hemolysate, prepared from human erythrocytes by the method of Georgatsos,[10] was added to the preparation to insure complete prothrombin conversion after recalcification. As has been shown,[10] hemolysate by itself has only weak thromboplastin activity but will bring about prothrombin consumption in a manner similar to the action of platelet factor. Both require thromboplastinogen (antihemophilic globulin) as a cofactor for their activity.

RESULTS

As has been previously observed,[11] no significant change occurred in labile factor during the entire course of dicumarol therapy. There also was no prolongation of the thrombin time, indicating that no heparinoid substances were evolved such as occur in varying types of liver disease. As Hurn and associates[12] have shown, the serum antithrombin activity gradually increased during the administration of dicumarol and decreased abruptly or gradually when the drug was discontinued.

Figs. 1 and 2 are typical of the free prothrombin time, total prothrombin time, and thrombin production curves obtained in patients receiving dicumarol. The prolongation of the free prothrombin time was apparent within 24 hours and reached a maximum level within 48 to 72 hours after the start of therapy. The prolongation of the total prothrombin time lagged 24 to 48 hours and then roughly paralleled the free prothrombin time.

The free prothrombin time of plasma determined after adding serum to it remained normal for 48 to 72 hours and then became prolonged. There was, however, no linear correlation between the free prothrombin time of plasma alone and the free prothrombin time of plasma to which serum had been added. As can be seen in Figs. 1 and 2, the correction of the total prothrombin time

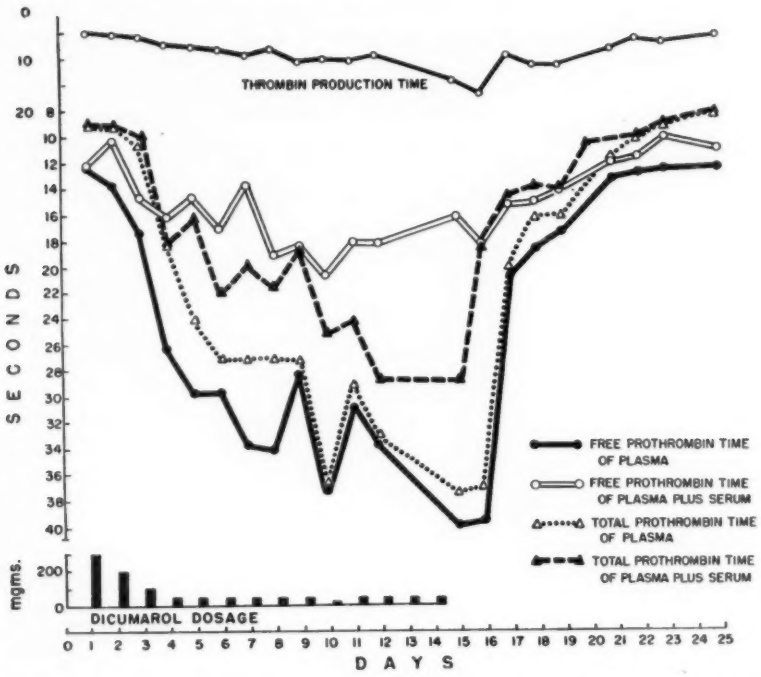

Fig. 1.—The thrombin production time, free prothrombin time, and total prothrombin time curves on one patient receiving dicumarol therapy.

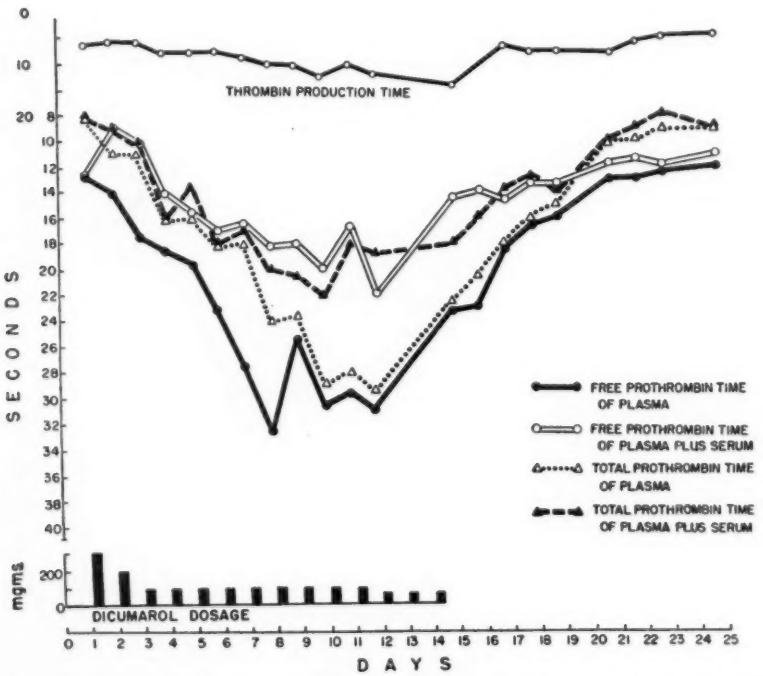

Fig. 2.—The thrombin production time, free prothrombin time, and total prothrombin time curves on a second patient receiving dicumarol therapy.

of plasma was greater than that of the free prothrombin time for 48 hours. Subsequently, the correction of the total prothrombin time, with few exceptions, was less than that of the free prothrombin time until discontinuance of the dicumarol. Percentagewise, the correction of the total prothrombin time by serum in the midst of therapy is almost negligible.

Thrombin production was slightly depressed though not until the free prothrombin time was markedly prolonged. No depression was noted if the free prothrombin time remained shorter than 20 seconds.

DISCUSSION

Owren[3] and later Koller[4, 5] modified their determinations of "prothrombin" by the addition of serum, stating that under dicumarol therapy the prolongation of the free prothrombin time is due, to a large extent, to a decrease in Factor VII. That a Factor VII deficiency is produced may be indicated by the shortening of the prothrombin times on the addition to the plasma of normal serum, a good source of the factor. If this were entirely the case, one would expect that the correction of the total prothrombin time would be proportional or at least comparable to the correction of the free prothrombin time as it is in the case of a true Factor VII deficiency.[9] In the present study, however, after 48 hours of therapy the total prothrombin time could be corrected to a lesser extent than the free prothrombin time. This discrepancy persisted until dicumarol administration was stopped.

Quick and his associates have shown that little or no thrombin can be produced from the plasma of individuals with a true congenital hypoprothrombinemia.[13] In our study, treatment of the plasma of dicumarolized patients in a manner similar to that described by Quick demonstrated only a slight decrease in thrombin production. Furthermore, this did not occur unless the free and total prothrombin times were markedly prolonged. When the free prothrombin time of dicumarol plasma was the same as the free prothrombin time of Quick's subjects, no appreciable decrease in production of thrombin was evident. These observations suggest that the prolongation of the prothrombin time of dicumarol plasma does not reflect a true hypoprothrombinemia.

If dicumarol does not cause hypoprothrombinemia, several possibilities present themselves to explain the prolongation of the Quick prothrombin time. One is that some factor is evolved which, in the determination, interferes with the conversion of prothrombin to thrombin, or, two, that some factor necessary for the conversion is lacking. That such factors exist have, as yet, not been demonstrated conclusively. A third possibility is the production of a modified prothrombin, as proposed by Lein and Lein,[14] which does not convert to thrombin in the physiologic balance as found in plasma but which will form thrombin in a nonphysiologic system. If, as Seegers and co-workers[15] have postulated, Factor VII is derived from or is part of the prothrombin complex, alteration of the prothrombin molecule could also result in an apparent demonstrable decrease in this factor. A variation in the solubility of such an

altered protein would explain the slight depression of thrombin production observed in our cases. The proof of the existence of such a changed protein might lie in electrophoretic studies.

SUMMARY

The effect of dicumarol on various factors of blood coagulation was studied in nine patients receiving the drug.

The observations indicate that dicumarol does not cause a true hypoprothrombinemia.

The production of an altered or abnormal prothrombin by dicumarol is suggested as an explanation for the prolonged Quick prothrombin time.

I wish to express my thanks to Mrs. Elizabeth Allie for her technical assistance and to Dr. Armand J. Quick and Miss Clara Hussey for reviewing the manuscript.

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NEUTRALIZATION OF HEPARIN IN VIVO WITH PROTAMINE; A SIMPLE METHOD OF ESTIMATING THE REQUIRED DOSE

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DOGS connected to an artificial heart-lung machine occasionally develop a postoperative syndrome characterized by shock and the development of a bleeding tendency.[1] We have obtained evidence which indicates that the use of excessive amounts of protamine may be, in part, responsible for this picture.

The protamine is administered to neutralize the effect of heparin, which must be used to prevent activation of the coagulation mechanism as the blood passes over foreign surfaces. At the conclusion of the perfusion it seems wise to return the clotting time to normal as quickly as possible in view of the wide incisions, the major blood vessels severed, and the drop in platelet count that occurs.[2] This relative thrombocytopenia has multiple causes and is probably the major factor responsible for the bleeding tendency mentioned above.

Protamine doses are usually calculated very roughly on the basis of previous experience, and although it has long been known that excess amounts of protamine have an anticoagulant effect, many surgeons use doses of protamine which border on the toxic. Excess protamine can be harmful in at least three ways. It can prolong the clotting time; it can lower the platelet count;[2, 3] and it can send the dog into shock. Dogs whose blood has been passed through an extracorporeal pump are peculiarly susceptible to the hypotensive action of protamine.[4]

In view of the toxic effects of excess protamine and the possibility that inaccurate neutralization of heparin might contribute to the hemorrhagic syndrome, we developed a procedure which enables us to estimate simply the minimum required dose of protamine. The procedure is essentially a modification of the protamine titration test of Allen,[5] but the requirements of the present situation permit an approach simple enough to be practical in the operating room.

METHODS

All experiments were performed on apparently healthy mongrel dogs. Heparin (Upjohn Heparin Sodium, 10 mg. per milliliter) was given intravenously in doses of 2.5 mg. per kilogram. The protamine required was estimated approximately one hour later.

Dilutions of protamine (Lilly 1 per cent) were made with saline from the same lot which was to be injected. Concentrations from zero to 50 mcg. at intervals of 5 mcg. in a volume of 0.1 milliliter were placed in a row of 12 × 75 ml. test tubes. One milliliter

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of blood was added to each 0.1 ml. of protamine solution. The tubes were then inverted once to mix their contents and were kept at room temperature. They were inspected as outlined below to see which ones had clotted.

RESULTS

An example of the clotting times of heparinized blood added to a row of protamine tubes is shown in Fig. 1. The shape of the curve is characteristic and has three important aspects. The transition from almost incoagulable blood due to excess heparin to a normal clotting time occurs in adjacent tubes; a level plateau of normal clotting times follows; and the tubes with the highest amounts of protamine again show a prolonged clotting time.

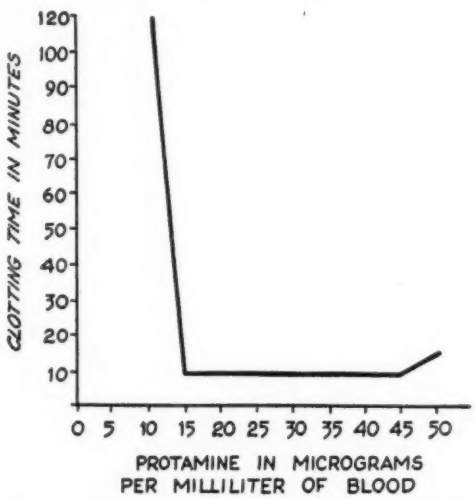

Fig. 1.—The clotting times of heparinized blood added to various concentrations of protamine.

A priori one might have expected a point of minimum clotting time where heparin was exactly neutralized. The experiment was repeated, with use of three tubes at each concentration of protamine and determination of their clotting time in sequence in the manner of the Lee-White clotting time. This portion of the experiment was carried out in a water bath at a temperature of 37° C. In the range where the plateau occurred, the third tubes all clotted at the same moment, proving that the plateau is indeed level. The best explanation for this level portion of the curve is that the heparin is neutralized in the tube with the least amount of protamine which brings the clotting time to normal, but that excess protamine does not affect the clotting time until relatively higher concentrations are present.

The abrupt transition from the tube with excess heparin to the tube which has the minimum effective amount of protamine means that the latter tube can be identified by inspecting the row of tubes only once somewhere near the end of a normal clotting time. Experience has shown that 15 minutes is the best time interval. Knowing the amount of protamine that will just neutralize the heparin effect in 1 ml. of blood, one must multiply this figure by the estimated blood volume of the dog to find the required dose of protamine.

Table I shows the results of two successive titrations done on a dog who was given protamine in accordance with our usual routine prior to this study. In this early experiment protamine concentrations were at intervals of 10 mcg. The first titration, done after the initial dose of protamine, showed that the dog had already received enough, inasmuch as he had a normal clotting time in the tube with no added in vitro protamine. The second titration, after a second injection of protamine, showed that we had come within 10 mcg. per milliliter of prolonging the dog's clotting time due to excessive protamine.

TABLE I. RESULTS OF TITRATIONS IN CASE GIVEN EXCESSIVE AMOUNTS OF I.V. PROTAMINE

Protamine (γ/ml.)	0	10	20	30	40	50
After first dose of I.V. protamine	X	X	X	X	0	0
After second dose	X	0	0	0	0	0

X equals clotted. 0 equals no clot.

Table II shows the results with the method as currently used. The initial titration shows a need for 15 mcg. of protamine per milliliter. In a dog with a blood volume of one liter, this would be 1.5 ml. of the 1 per cent solution. Actually the dog received 2 ml. plus a transfusion of heparinized blood. The second titration demonstrates a need for still more protamine, which was given. The third row of tubes shows a normal clotting time in the tube with no added in vitro protamine, yet the anticoagulant effect of excess protamine is not apparent until a considerable amount of in vitro protamine is added.

TABLE II. RESULTS OF TITRATIONS IN CASE GIVEN I.V. PROTAMINE ACCORDING TO PRESENT METHOD

Protamine (γ/ml.)	0	5	10	15	20	25	30	35	40	45	50
Before I.V. protamine	0	0	0	X	X	X	X	X	X	X	X
After first dose	0	0	X	X	X	0	0	0	0	0	0
After second dose	X	X	X	X	X	X	X	X	0	0	0

DISCUSSION

Since heparin effect must be neutralized after an artificial heart-lung perfusion, and since excessive amounts of protamine are toxic, a simple method is needed to indicate the necessary dose of protamine.

Such a method must take into consideration the following variables which affect the amount of protamine needed to neutralize a given amount of heparin. (1) Considerably less protamine is needed to neutralize the same amount of heparin in vivo than in vitro. (2) Protamine and heparin preparations vary considerably in their activity. (3) As time elapses after heparin is injected, less and less protamine is needed. (4) Heparin effect varies with the platelet count.[6] Platelets have an antiheparin substance,[7] and the lower the platelet count the greater the effect of a given amount of heparin. (5) Heparin is produced in increased amounts in animals under certain conditions,[8] and the possibility exists that its production might vary under the conditions of the operation.

Our method avoids these variables by making the protamine dilutions from the solution which will be injected and by adding to it blood taken from the dog just prior to the test.

The method is also simple enough to be practical for use in the operating room. The protamine dilutions can be made in the laboratory during the operation. To perform the actual titration, one need only add 1 ml. of blood from a syringe to each tube. At the end of fifteen minutes, the tubes are tilted to find the one with the least amount of protamine that has clotted. The sharp end point which separates this tube from the adjacent one with excess heparin makes it possible to perform the test at room temperature, since the end point does not differ from that obtained in a water bath at 37° C.

Preliminary observations in human cases show that the method works as effectively in humans as in dogs.

SUMMARY

1. Excess protamine in part explains the syndrome of shock and a bleeding tendency that occurs in dogs whose blood has been perfused through an artificial heart-lung machine.

2. A simple method is described which provides an estimate of the minimal amount of protamine needed to neutralize the heparin in the animal at any given time.

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THE ROLE OF TRANSFUSED LEUKOCYTES IN EXPERIMENTAL BACTEREMIA OF IRRADIATED RATS

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INTRODUCTION

RECENT studies in our laboratory have shown that appreciable numbers of leukocytes persist for short periods in the circulation of irradiated, leukopenic rats following cross-circulation with normal rats.[1] The removal mechanism of these transfused leukocytes remains an enigma, but our studies suggested that these cells were not destroyed but remained viable in noncirculating sites. To test this hypothesis we sought to use some test of leukocyte function as an index of leukocyte viability after cross-circulation. We have demonstrated following intravenous inoculation with *Endamoeba coli* that irradiated rats have a significantly higher level of bacteremia than do normal rats.[2] This impaired ability to remove bacteria did not seem to be related to reticuloendothelial function, and we postulated that deficiency of leukocytes or some plasma factor, such as properdin,[3] might explain the impaired bacterial removal by the irradiated rat. The present report concerns observations on the effect of the cross-circulation procedure on experimental bacteremia.

METHODS

The strain of *E. coli* used was the same used in previous experiments, and the general method of study of bacteremia was identical.[2] After the experimental procedures described below, 0.5 ml. of a 4-hour broth culture of the test organism, containing approximately 200,000,000 viable organisms, was injected intravenously through a polyethelene catheter placed in the femoral vein of the animal. Heparin, 0.2 ml., was injected to prevent blood coagulation. Blood samples from a polyethelene catheter in the femoral artery were obtained at intervals of ½, 1, and 2 hours after injection. These samples were diluted with saline in tenfold increments, pour plates were prepared in blood-agar base, and the bacterial colonies were counted after 48 hours incubation at 37° C.

Rats used in all experiments were Sprague-Dawley strain males, weighing 250 to 350 grams.

Anesthesia was obtained by intraperitoneal injection of 12 mg. of pentobarbital sodium, supplemented as necessary during the procedures.

The following experimental groups of rats were included in the present study:

Group I.—Normal rats.

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Group II.—Irradiated rats. The animals were subjected to 800 roentgens (250 kv.; 15 Ma; Thoreus filter; 50 cm. tube distance) total body exposure 72 hours before study. This dosage is reported by Cronkite and Brecher[4] as almost 100 per cent lethal.

Group III.—Irradiated rats following cross-circulation with normal rats. The irradiation was performed as in Group II. Cross-circulation was effected by the method described by Brodish and Long.[5] Briefly the method consists of outflow from the artery of one animal into the vein of the opposite animal through a system of plastic tubes and cups. The apparatus allows accurate measure of the volume of blood transferred, and in these experiments, 60 to 120 ml. of blood (200 to 400 per cent circulatory mixing) was transferred in 30 to 90 minutes. After cessation of cross-circulation, the bacterial removal was immediately studied after a preliminary culture was taken to check the sterility of the procedure.

Group IV.—Normal rats that served as the cross-circulation partners for the irradiated rats of Group III.

Group V.—Zymosan-treated rats. Normal rats were given intraperitoneal zymosan,* 100 mg. per kilogram of body weight, 18 hours before the bacteremia studies were performed.

Group VI.—Irradiated rats subjected to replacement transfusion with normal heparinized rat blood from which the leukocytes had been partially removed by mechanical means. Sterile blood was obtained by direct cardiac puncture of normal rats and drawn into syringes lightly coated with heparin solution. After centrifugation the plasma was aspirated and saved, the buffy coat was aspirated and discarded, and the red cells were recombined with the plasma. Leukocyte counts of the pooled reconstituted bloods varied from 500 to 1,700 cells per cubic millimeter. The blood was used for replacement transfusion of the irradiated rats within 4 hours of the time of collection. Replacement transfusion was performed by means of polyethylene cannulae in the femoral artery and vein. An animal was allowed to bleed freely from the artery, and the rate of blood loss was matched by injection of the artificially prepared leukopenic blood into the femoral vein catheter. Replacement of 20 ml., sufficient to give 80 per cent blood-volume exchange, was completed in 4 to 10 minutes. After preliminary blood cultures were taken to insure the sterility of the procedure, the animals were subjected to the standard bacteremia studies.

Appropriate normal or irradiated control animals were included with all experiments in order to detect any biologic variability of the test organism or of the rats used. Statistical analyses were performed using a test of ranks,[6] since the marked variation of bacteremia levels in the irradiated rats precluded the use of conventional statistical methods employed when results show a typical mean distribution.

RESULTS

Colony counts of the 30-minute blood samples from all animals in six different groups are recorded in Table I. In Figs. 1 to 4, the linear two-hour disappearance of organisms from the blood stream of each rat in Groups III to VI is plotted with the range of bacteremia levels in normal rats (heavily stippled) and irradiated rats (lightly stippled) included for comparison. The data are expressed as percentage of bacteria retained in the blood, with the 100 per cent value estimated from the size of bacterial inoculum and the blood volume. The logarithmic plot of the ordinate on a linear scale is necessitated by the wide range of bacterial counts involved.

The clear-cut difference in the colony counts of the normal and irradiated rats (Groups I and II) is quite evident from inspection of Table I, with a calculated $P < 0.001$ at each of the three time intervals.

*Obtained from Standard Brands, Inc., Stamford, Conn.

Cross-circulation between irradiated and normal rats caused a significant improvement ($P < 0.01$) in the ability of the irradiated rat to remove injected bacteria (Fig. 1). Results obtained in bacteremia studies of the normal rats that served as partners during the cross-circulation are plotted in Fig. 2. The results suggested that the procedure had caused slight impairment in their ability to remove bacteria, but analysis of the data revealed no significant difference between the bacteremia in these rats and the controls (Group I).

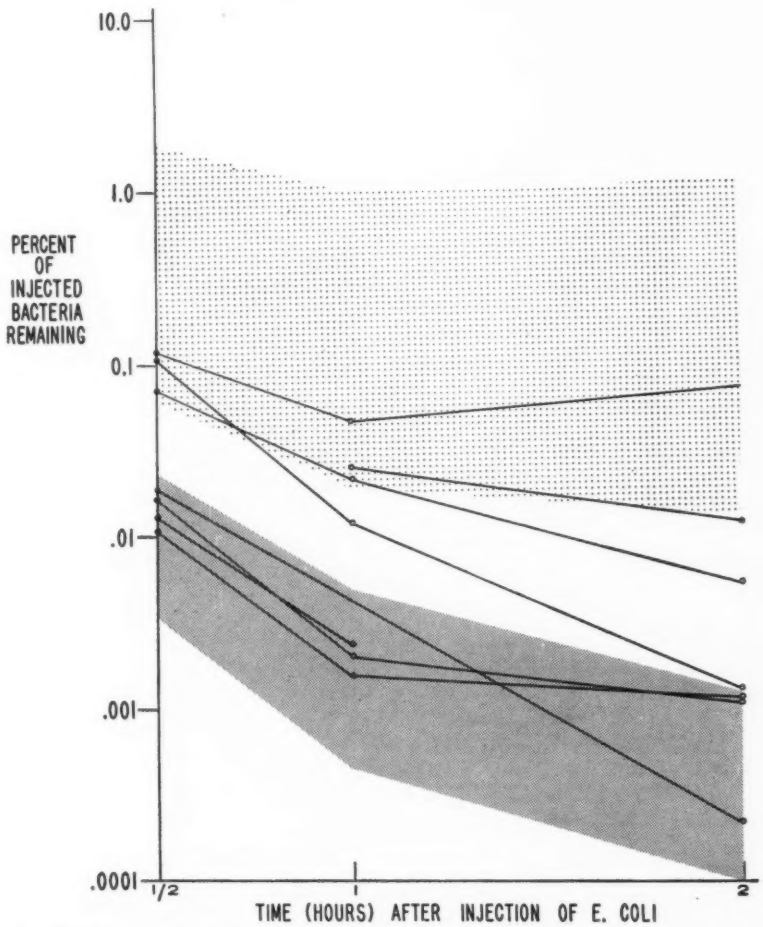

Fig. 1.—The per cent of injected bacteria remaining in the blood for each of the eight irradiated rats after cross-circulation is shown by a solid line. The range of bacteremia levels for normal and irradiated rats which have not been cross-circulated is represented by the heavily and lightly stippled areas, respectively.

The changes in leukocyte count resulting from the cross-circulation between normal and irradiated rats have been reported by us in detail.[1] During cross-circulation the arterial leukocyte counts of the irradiated rats rose moderately, while the arterial leukocyte count of the normal rat tended to decrease. Prolonged mixing of the circulations failed, however, to produce

TABLE I. COLONIES PER MILLILITER OF BLOOD OF THE EXPERIMENTAL GROUPS OF RATS 30 MINUTES AFTER INTRAVENOUS INJECTION OF E. COLI

GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
610	8,100	1,300	710	180	2,580
1,030	8,900	2,080	750	450	5,700
1,110	12,500	2,960	1,110	460	6,500
1,130	14,600	3,400	7,500	600	7,000
1,530	18,400	8,100	8,900	1,090	9,100
1,600	18,600	14,000	13,100	1,270	11,400
1,720	29,800	15,200		1,920	12,800
1,750	32,800			9,500	13,900
1,840	332,800				13,900
1,990	93,000				39,400
2,600	131,000				129,000
4,300	144,000				
	161,000				
	182,000				
	348,000				

TABLE II. LEUKOCYTE COUNTS OF THE RATS INVOLVED IN CROSS-CIRCULATION STUDIES (GROUPS III AND IV)

BEFORE CROSS-CIRCULATION	IMMEDIATELY AFTER	½ HOUR AFTER	1 HOUR AFTER	2 HOURS AFTER
		Irradiated Rats (Group III)		
150	4,200		700	400
50	2,500	1,200	700	200
350	1,550	1,200	300	300
50	4,650	1,050	800	200
0	6,120	1,250	1,250	500
50	2,250	850	350	
50	6,900	1,600	1,400	350
100	9,250	1,550	650	400
		Normal Rats (Group IV)		
8,550	6,450		12,900	11,750
4,450	6,450	5,100	3,050	8,200
12,450	7,900	7,600	6,800	
16,950	8,200	7,350	5,700	8,800
9,900	4,750	5,200	4,450	4,900
16,200	12,300	12,300	17,000	12,750
16,300	13,200	7,650	6,700	

TABLE III

LEUKOCYTE COUNT OF REPLACEMENT BLOOD	BACTERIAL COLONY COUNT OF THE IRRADIATED RATS REPLACED, ½ HOUR AFTER INJECTION
500	39,400
500	129,000
1,000	12,800
1,000	9,100
1,600	7,000
1,600	13,900
1,600	5,700
1,600	2,580
1,700	6,500
1,700	13,900

equalization of the counts of the two animals. After cessation of cross-circulation, the leukocyte counts of the irradiated rats fell progressively, reaching the base line at 1 to 2 hours. The counts of the normal rats tended to return to normal levels. Arterial leukocyte counts of the animals used in these experiments (Groups III and IV) are shown in Table II. Leukocyte counts were obtained before and after the period of cross-circulation and at the times of collection of the blood samples for the bacteremia study.

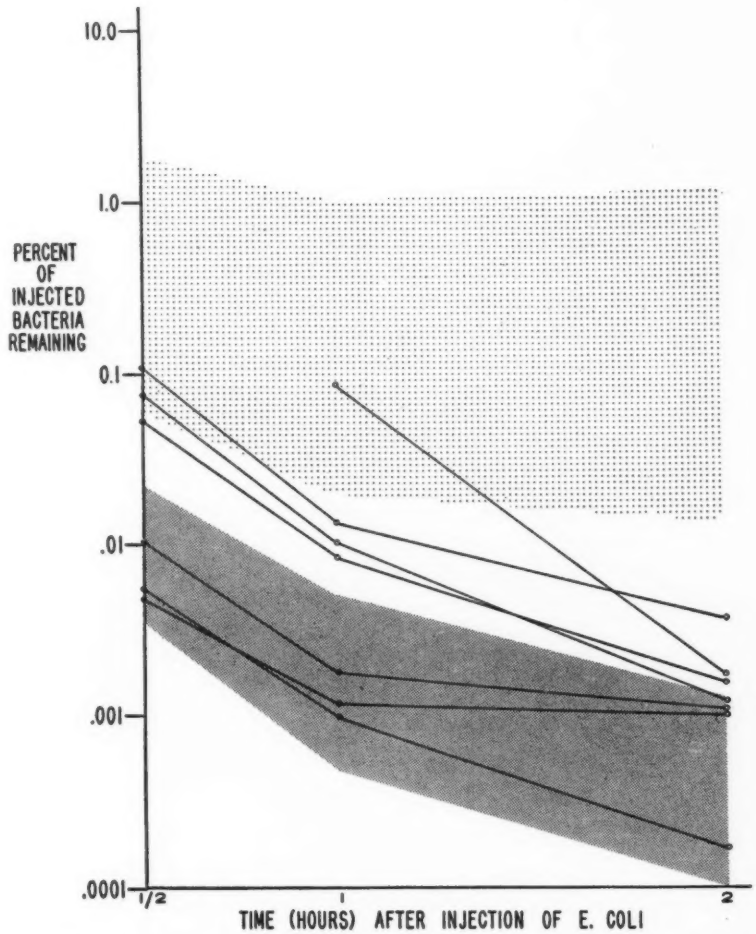

Fig. 2.—The per cent of injected bacteria remaining in the blood for each of seven normal rats which served as donors for cross-circulation with the irradiated rats is shown by a solid line. The range of bacteremia levels for normal and irradiated rats which have not been cross-circulated is represented by the heavily and lightly stippled areas, respectively.

Zymosan, a material obtained from yeast, has been reported by Pillemer and Ross[3] to cause a marked drop in properdin titer after intraperitoneal injection into rats. The zymosan-treated animals (Group V) demonstrated no impairment in their ability to remove bacteria (Fig. 3).

When irradiated rats were subjected to replacement transfusion with normal rat blood from which the leukocytes had been removed (Group VI), there appeared to be some improvement in bacterial removal, but the difference was of only minimal statistical significance (Fig. 4). Comparison of these animals (Group VI) with the irradiated controls (Group II) revealed at the one-half-

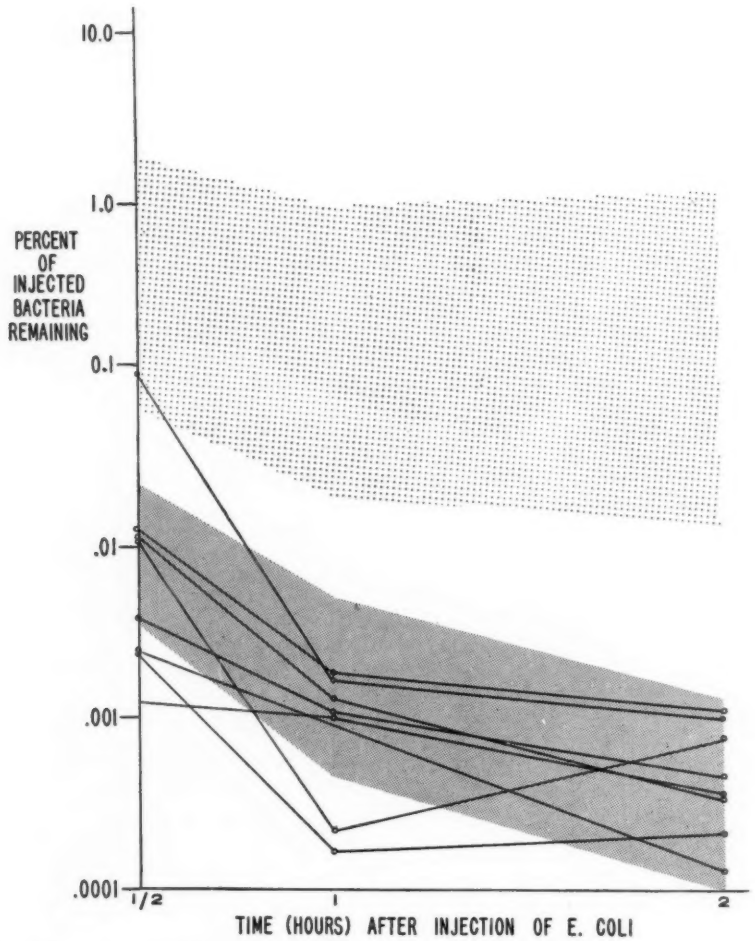

Fig. 3.—The per cent of injected bacteria remaining in the blood for each of eight normal rats treated with zymosan is shown by a solid line. The range of bacteremia levels for normal and irradiated rats which have not been cross-circulated is represented by the heavily and lightly stippled areas, respectively.

hour period after injection of bacteria $P < 0.05$; at one hour, $P < 0.05$; and at two hours, $P > 0.05$. From inspection of the data it appeared that when the white cells were most completely removed from blood used for replacement transfusion, the level of bacteremia in the irradiated rats was not altered. With less complete leukocyte removal, the level of bacteremia appeared to be

lowered (Table III). Statistical proof of this relationship could not be established in this relatively small number of studies, and it was not considered feasible to obtain a considerably larger number of observations.

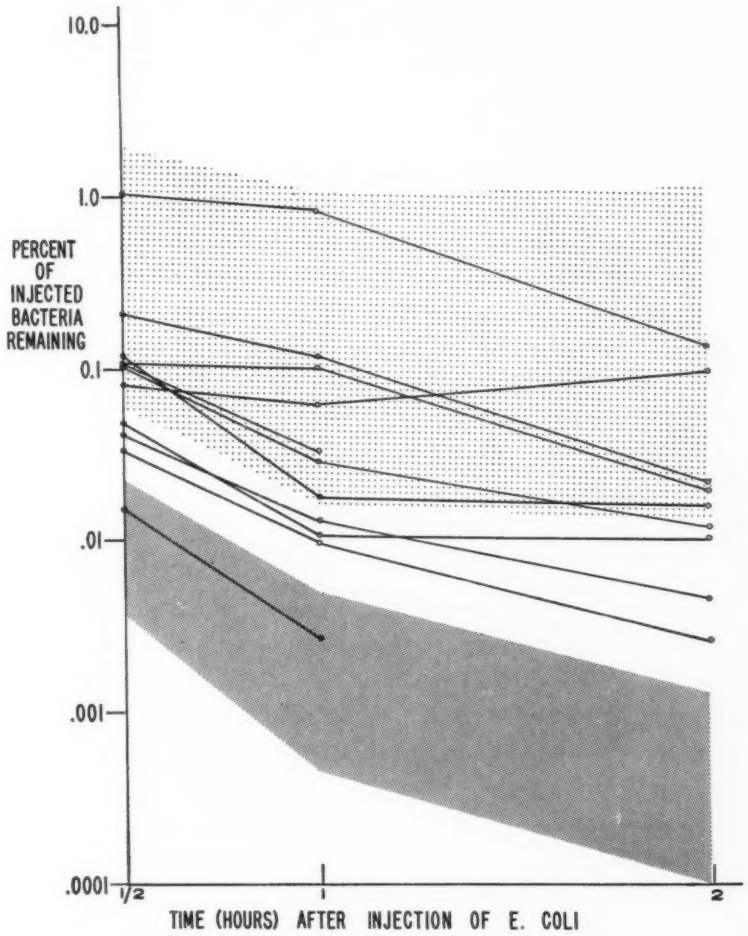

Fig. 4.—The per cent of injected bacteria remaining in the blood for each of eleven irradiated rats after replacement with leukopenic blood is shown by a solid line. The range of bacteremia levels for normal and irradiated rats which have not been cross-circulated is represented by the heavily and lightly stippled areas, respectively.

DISCUSSION

It has been demonstrated that rats subjected to severe irradiation are unable to remove injected *E. coli* from their blood as efficiently as normal rats. This defect in bacterial removal was significantly improved after cross-circulation of irradiated with normal rats. The beneficial results of the cross-circulation indicated that reticuloendothelial function was not impaired and that the improvement resulted from a transfer of some substance in normal blood into the irradiated rats. The factor present in the normal blood could be some plasma factor or leukocytes.

Properdin, a bactericidal plasma factor which might logically be implicated in the removal of the *E. coli,* did not seem to be of importance. Injection of zymosan into normal rats in an attempt to produce properdin deficiency did not adversely affect bacterial removal. No properdin assays were done, however. We attempted to exclude all plasma factors by replacement transfusion of the irradiated rat with heparinized fresh blood, containing everything except leukocytes, from normal rats. In these experiments the buffy coat was simply aspirated and discarded, leaving 500 to 1,700 leukocytes per cubic millimeter. Questionable improvement in the ability of the irradiated rat to remove bacteria was noted. There was suggestive evidence that the leukocytes still remaining in the replacement blood were responsible for the beneficial effect of this treatment. One might have expected, also, that the improvement noted after replacement transfusion would have been greater than that after cross-circulation if a plasma factor was involved. Cross-circulation could only increase the plasma level by a 50:50 mixing of the two animals unless there was very rapid new formation of the protein in the normal rat, while replacement would presumbly increase the level of a plasma factor to 70 to 80 per cent of normal. We believe it unlikely that a substance in plasma was responsible for correction of the defect in bacterial clearance noted in the irradiated rat.

More probably, the leukocytes were the source of the improvement noted after cross-circulation, and the leukopenia of the irradiated rat was responsible for the higher level of bacteremia in irradiated animals. If surface phagocytosis[7] by the transfused leukocytes is the mechanism of improvement, one must presume that transfused leukocytes are not immediately destroyed. Much evidence has been presented that transfused leukocytes are not viable but are trapped and destroyed by the lung. Certainly rabbit leukocytes labeled with radioactive phosphorous collected from peritoneal exudate, washed with saline, and injected intravenously are lysed.[8] However, the concept of massive destruction of leukocytes by normal lung, as derived by Bierman and associates[9] from simultaneous pulmonary artery—pulmonary vein leukocyte counts in man, is not consistent with the relatively long lifespan of the leukocyte as determined experimentally by radioactive phosphorous-labeled DNA.[10, 11]

Osgood[12] has determined that probably less than 1 per cent of leukocytes in the body are in the circulating blood, while the remainder are in stores elsewhere. The rapid disappearance of transfused leukocytes in the irradiated rat, then, need not represent destruction but simply redistribution of leukocytes. Brecher, Wilbur, and Cronkite[13] have obtained evidence that such leukocytes are viable and may migrate to a focus of infection. One probable site of distribution of leukocytes is along the capillary walls, a site that has been particularly evident in certain of the in vivo capillary microscopy studies of Vejlens[14] and in the studies and motion pictures of Fulton and co-workers.[15]

During our cross-transfusion studies, at least 10,000 leukocytes per cubic millimeter were transfused into the irradiated rat, of which about 2,000 per cubic millimeter were granulocytes. The bacteremia in irradiated rats was of a level of about 10 organisms per cubic millimeter. If any appreciable number of the transfused granulocytes were viable in capillaries, phagocytosis by these cells could easily account for the decreased bacteremia noted after cross-circulation of the irradiated rats.

SUMMARY

1. Irradiated rats exhibited higher levels of bacteremia than normal rats, following intravenous inoculation of a culture of *E. coli*.

2. Cross-circulation between irradiated and normal rats significantly lowered the bacteremia in irradiated rats.

3. Injection of zymosan in normal rats, in an attempt to deplete the bactericidal serum protein, properdin, did not appreciably affect the bacteremia.

4. Replacement transfusion of the irradiated rat with normal blood from which the buffy coat had been removed caused some diminution of the level of bacteremia, but the most probable explanation for this seemed to be incomplete leukocyte removal from the normal blood.

5. It is postulated that the leukopenia of the irradiated rats was responsible for the increased bacteremia and that the transfused leukocytes corrected the defect.

We would like to express our appreciation to Mrs. Elizabeth Maloney and to Miss Mary Chaltas for technical assistance. Dr. Chu H. Chang and Miss Anna Allen performed the irradiation. Dr. Colin White was most helpful in statistical analysis of the data.

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A STUDY OF THE CORRELATION OF ANTIGEN PHAGOCYTOSIS AND THE SPLENIC HISTOLOGIC REACTION WITH ANTIBODY FORMATION IN PROTEIN-DEPLETED RATS

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A SERIES of investigations in this department has shown that severe dietary protein deficiency results in a marked inhibition of antibody response in rats following the intravenous injection of antigen. This decreased response has been attributed to the inability of the animals to synthesize new antibody gamma globulin because of a deficiency of available amino acids following a prolonged low protein intake and depleted protein reserves.[1-10] These observations in lower animals have been substantiated in man by studies of antibody response during protein deficiency.[11-15]

This study was undertaken to investigate some of the steps of antigen localization and degradation and antibody formation in normal and protein-depleted rats. The main purpose was to elucidate further the mechanism by which protein deficiency modifies the immunologic response to a single intravenous antigen injection and to compare the effects of protein deficiency with other factors inhibiting the immune response, which have been studied in this laboratory and elsewhere. It seemed possible that this experimental approach might, in addition, contribute to the understanding of the site and mechanism of antibody formation. Particular emphasis was given to (1) the rate of phagocytosis of labeled antigen (removal from the blood), (2) antigen distribution in various tissues, (3) disappearance of labeled antigen from the tissue, the rate of excretion of the label, and (4) the cellular response to the antigen (mainly in the spleen). The experimental methods used by Fitch and associates,[16] in this laboratory, seemed particularly suitable for the present experiments.

MATERIAL AND METHODS

Experimental Plan.—Four experiments were performed, each with the same general plan. Young adult male Sprague-Dawley albino rats with initial weights of 180 to 250 grams were used. Half of the animals were fed a low protein diet and the other half a control diet until the weight loss of the animals in the former group was between 27 and 38 per cent (Table I). The animals were then injected intravenously with a labeled antigen. Subgroups of three animals from each group were bled and sacrificed at 15 and 60

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minutes and at 1, 4, and 6 days after antigen injection. Their organs and tissues were used for histologic and radioautographic studies to determine the distribution of the antigen. Noninjected animals from each diet group were also sacrificed at each time interval to serve as controls. Subgroups of three animals from each diet group were bled at 5, 15, 30, and 60 minutes after injection to study the early rate of disappearance of the antigen from the blood. Three protein-deficient and three control rats were kept in metabolism cages throughout each experiment to measure the urinary excretion of I^{131}.

TABLE I. WEIGHT VARIATIONS IN PROTEIN-DEPLETED AND CONTROL ANIMALS

EXP. NO.	DIET PERIOD (DAYS)	DIET	WEIGHT LOSS OR GAIN (GM.)	WEIGHT LOSS OR GAIN (PER CENT)
1	52	4E	-81	-34
	52	4C	+33	+13
2	42	4E	-66	-31
	42	4C	+25	+31
3	70	4E	-93	-38
	70	4C	+59	+25
4	42	4E	-56	-27
	42	4C	+26	+13

There were certain deviations from this routine. In the first experiment, six additional nondepleted animals were injected with sheep erythrocytes alone. In the second experiment, carbon particles were injected simultaneously with the labeled antigen to determine whether the phagocytized radioactive vaccine was localized in the same cells as inert particles and to mark these cells for the remainder of the experiment. In the third experiment, sheep erythrocyte stromata labeled with I^{131} were used as antigen, instead of typhoid vaccine, and the sacrifice intervals were 60 minutes, 8 hours, and 1, 4, and 6 days. In the fourth experiment, sheep erythrocyte stromata in doses larger than usual were used, and the sacrifice intervals were 60 minutes, and 1, 4, and 6 days. Only the histologic reaction of the spleen and the antibody response were studied in this experiment.

Diets.—The diets were essentially those called 4E and 4C by Wissler and co-workers,[3] with dextrin substituted for cornstarch. Each rat was given 15 Gm. per day until 2 to 7 days prior to the beginning of the experiment, after which the daily ration was mixed with an equal amount of tap water containing 0.5 per cent of Lugol's solution to "saturate" the thyroid with iodine and prevent its uptake of I^{131}. The same amount of Lugol's solution was added to the drinking water.

Antigen Preparation and Antibody Determination; Antigen Labeling; Measurement of Radioactivity.—The methods for antigen preparation, antibody determination, antigen labeling, and measurement of radioactivity have been described previously by Cannon and co-workers.[1, 2] and by Fitch and associates.[16] Sheep erythrocyte stromata were prepared according to the method of Ingraham[17] and labeled with I^{131} with the use of the method described by Fitch and co-workers.[16]

Histologic Techniques.—The radioautographic techniques have been described.[16] Organs taken for histologic study were imbedded in paraffin and stained with methyl green-pyronine, with use of the Popper modification[18] of the Unna method[19] as follows: 0.25 Gm. pyronine and 0.75 Gm. methyl green were dissolved in 100 ml. phosphate buffer (52.5 ml. M/5 Na_2HPO_4 and 47.5 ml. M/10 citric acid), and 0.5 ml. of a 0.5 per cent phenol solution and 2.5 ml. of a 1 per cent resorcin solution were added. After deparaffinization the sections were stained for 10 minutes, differentiated shortly in water, quickly dehydrated in acetone, cleared in xylene, and mounted. Ribonuclease-treated control sections[13] were always compared with the experimental preparations. Only methyl green-pyronine stains were used for the histochemical determination of ribonucleic acid, since previous studies[16] showed close agreement with the azure B metachromasia.[20]

RESULTS

Because of experimental variations, some of the results of the different experiments will be reported separately. In the first experiment, at the end of the depletion period, 13 protein-depleted and 16 nondepleted animals were injected intravenously with 0.5 ml. of I^{131}-labeled *Salmonella typhi* vaccine and with 1.0 ml. of an unlabeled 0.5 per cent suspension of sheep erythrocytes at the same time. The activity of the injected dose of labeled antigen was 338,000 counts per minute. Six nondepleted animals were injected with sheep erythrocytes alone.

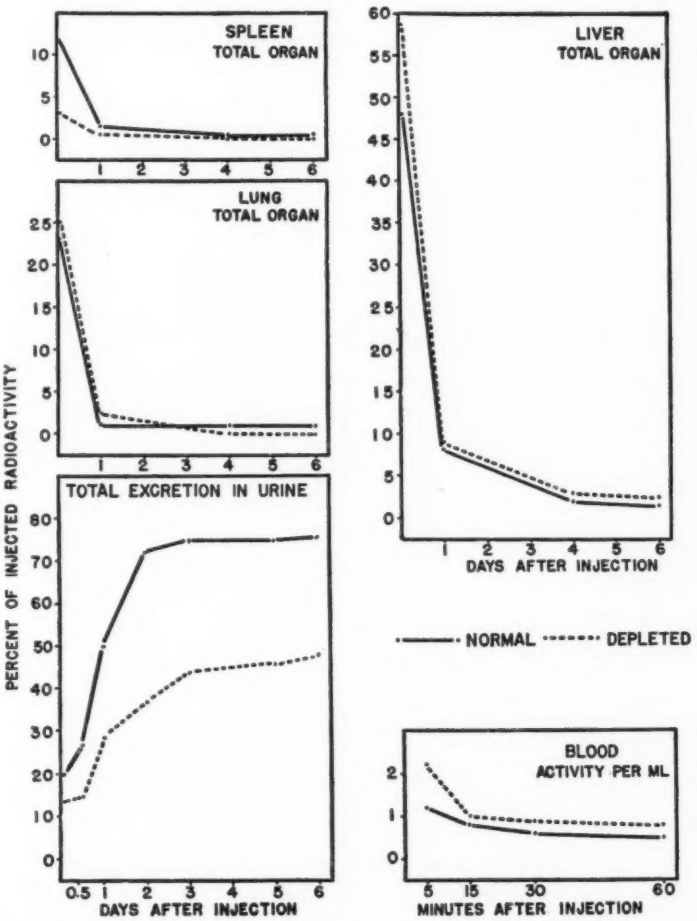


Fig. 1.—Experiment I. Radioactivity in various organs, urine, and blood following a single intravenous dose of I^{131}-labeled *S. typhi* vaccine in protein-depleted and control animals.

Radioactivity was determined in the liver, spleen, lungs, kidneys, and thymus. Samples of liver, spleen, lung, kidney, thymus, mesenteric lymph node, and bone marrow were taken for histologic study. The per cent of radioactivity per gram and per total organ in the liver, spleen, lungs, blood, and urine are recorded in Table II and Fig. 1. Antibody determinations are shown

in Fig. 2. The spleen showed a consistently higher level of radioactivity in the nondepleted animals until one day after antigen injection. This apparently indicates that the rate of phagocytosis by the reticuloendothelial system of the spleen was more efficient in the nondepleted than in the depleted animals. Although the difference in content of radioactivity per gram of tissue in these spleens is significant, much more striking is the difference in radioactivity per total organ. The spleens from depleted animals were smaller than those from normal animals (Fig. 2), and the early lower concentration of radioactive antigen can be explained by a diminution in the rate of phagocytosis. In contrast, the livers of the depleted animals may have had a somewhat more efficient phagocytic activity in the early period after antigen injection. The

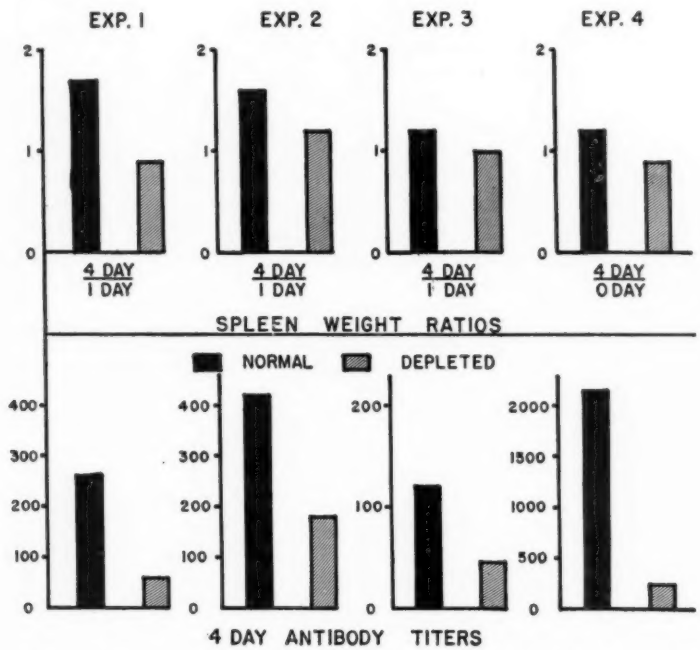


Fig. 2.—Ratio of weight of spleens at 1 day versus weight at 4 days after intravenous antigen injection in protein-depleted and control animals. Antibody determinations at 4 days after antigen injection in depleted and normal animals.

high levels of radioactivity in the lungs a few hours after injection were apparently due to embolization of small vessels by aggregates of antigen (Fig. 3). Disappearance of antigen from the blood stream was very rapid, indicating a swift removal by the reticuloendothelial macrophages. At 5 minutes after antigen injection, 75 per cent of the total radioactivity had already disappeared from the blood stream in normal animals and 77 per cent in depleted animals. The disappearance of the antigen from the total circulation in this and the following experiments was calculated from the measured disappearance per milliliter of blood, with use of the blood volume data recorded by Benditt and associates.[21] As reported in other experiments,[22] nonprotein-

bound I^{131} is excreted rapidly in the urine. Therefore, the high urinary concentration of I^{131} within the first 12 hours suggests a rapid degradation of some of the antigen. The far greater early urinary excretion of I^{131} in the normal rats could support the idea that the normal rat has a better capacity to digest antigen than the protein-depleted animal, at least to the point of removing the I^{131}. The kidney never contained more than 1.2 per cent of the injected dose of I^{131}, and Fitch and co-workers[16] reported that other organs, such as thyroid, bone marrow, etc., did not show appreciable amounts of radioactivity. Hence, counts on these organs were omitted in this and the following experiments. For the same reason the radioactivity of the feces was not determined. The absence of these analyses and the fact that I^{131} was not determined in the residual carcass make it impossible to ascertain with certainty the reason for the apparent greater excretion of I^{131} in the urine of the control rats. The available data are consistent with the conclusion that the greater excretion results in part from the greater breakdown (digestion) of antigen in the spleens of control rats.

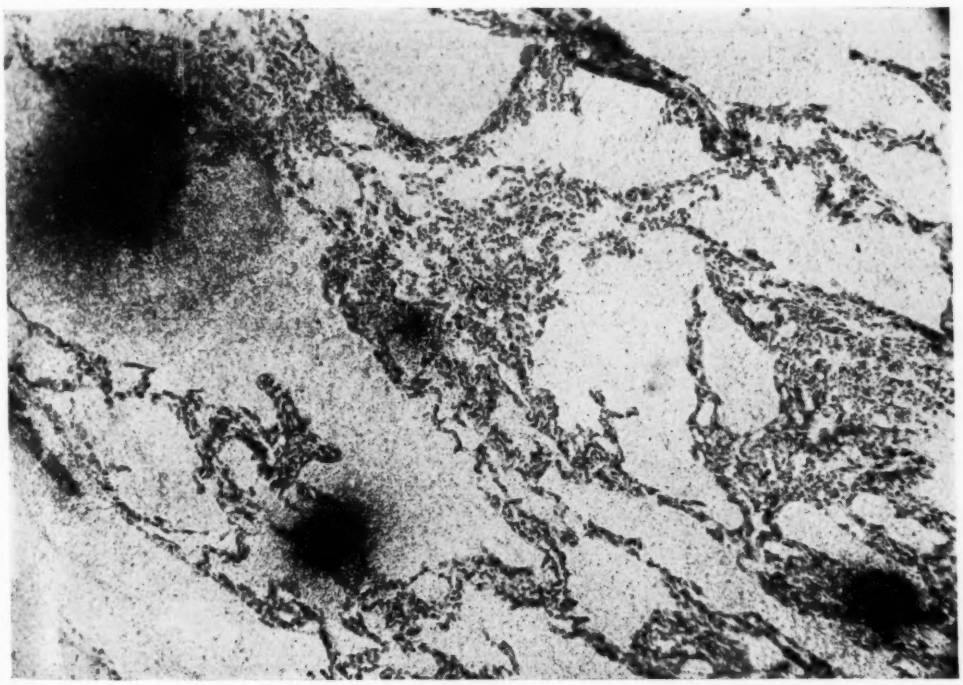


Fig. 3.—Distribution of I^{131}-labeled antigen in the lung of a normal rat 15 minutes after antigen injection. (Hematoxylin and eosin counterstain.)

In the second experiment, 14 protein-depleted and 15 nondepleted animals were used. The plan was similar to that of the previous experiment except that the animals received, in addition to 0.6 ml. of labeled *S. typhi* vaccine, 1.0 ml. of a thoroughly washed suspension of India ink in saline. This was done to determine whether the phagocytized I^{131}-labeled bacterial cells localized

in the same cells with carbon particles. Noninjected controls were studied from each diet group. The radioactivity of the injected antigen was 295,000 counts per minute. In general, the results of this part of the experiment were in agreement with those of the first. The spleens of protein-depleted and non-depleted animals showed the same early difference in rate of phagocytosis. At 5 minutes after antigen injection, 56 per cent of the antigen had disappeared from the blood stream of the normal animals and 63 per cent from the depleted animals. Again, the control rats excreted larger quantities of

TABLE II. DISTRIBUTION OF RADIOACTIVITY AND EXCRETION IN NORMAL AND DEPLETED ANIMALS FOLLOWING INJECTION OF LABELED *S. Typhi* VACCINE

PER CENT PER GRAM OF TISSUE						
	DEPLETED ANIMALS			NORMAL ANIMALS		
TIME	LIVER	SPLEEN	LUNGS	LIVER	SPLEEN	LUNGS
15 min.	7.7 ± 0.40	15.0 ± 2.07	21.0 ± 2.50	5.7 ± 1.92	20.7 ± 2.50	17.0 ± 2.44
60 min.	4.7 ± 1.34	11.7 ± 4.69	12.3 ± 2.34	5.3 ± 0.40	20.0 ± 0.0	5.6 ± 0.70
1 day	3.4 ± 0.12	1.6 ± 0.38	1.6 ± 0.38	0.6 ± 0.0	2.0 ± 0.0	0.5 ± 0.14
4 days	0.5 ± 0.25*	0.6 ± 0.0*	0.2 ± 0.31*	0.3 ± 0.0†	0.7 ± 0.08†	0.6 ± 0.28†
6 days	0.3 ± 0.0	0.7 ± 0.15	0.1 ± 0.0	0.1 ± 0.10	1.2 ± 0.37	0.1 ± 0.04

PER CENT URINARY EXCRETION		
TIME	DEPLETED	NORMAL
0- 6 hours	14.0 ± 8.0	20.3 ± 12.0
6-12 hours	1.0 ± --‡	7.0 ± 3.7
12 hours- 1 day	13.5 ± 4.9	23.3 ± 11.3
1- 2 days	8.5 ± 0.5	20.7 ± 13.7
2- 3 days	7.0 ± 3.0	4.3 ± 3.3
3- 4 days	-- --	-- --
4- 5 days	2.0 ± 1.0	1.2 ± 0.9
5- 6 days	2.5 ± 1.6	0.4 --‡

All results represent the mean from three animals and the standard error unless otherwise noted.

*Two animals.

†Four animals.

‡One animal.

TABLE III. DISTRIBUTION OF RADIOACTIVITY AND EXCRETION IN NORMAL AND DEPLETED ANIMALS FOLLOWING INJECTION OF LABELED *S. Typhi* VACCINE

PER CENT PER GRAM OF TISSUE						
	DEPLETED ANIMALS			NORMAL ANIMALS		
TIME	LIVER	SPLEEN	LUNG	LIVER	SPLEEN	LUNG
15 min.	9.7 ± 0.70	21.0 ± 3.74	6.3 ± 0.91	8.0 ± 0.57	27.3 ± 3.71	3.0 ± 0.0
60 min.	9.3 ± 0.70	17.3 ± 2.73	5.0 ± 0.57	7.0 ± 2.0	22.3 ± 2.34	3.1 ± 1.04
1 day	2.0 ± 0.0	5.0 ± 1.73	0.8 ± 0.04	0.8 ± 0.10	2.5 ± 0.97	0.2 ± 0.05
4 days	0.7 ± 0.0*	1.5 ± 0.50*	0.2 ± 0.10*	0.3 ± 0.0	1.1 ± 0.10	0.7 ± 0.0
6 days	0.5 ± 0.12	0.9 ± 0.12	0.1 ± 0.0	0.3 ± 0.0	0.9 ± 0.10	0.08 ± 0.05

PER CENT URINARY EXCRETION		
TIME	DEPLETED	NORMAL
0- 6 hours	8.83 ± 3.93	24.3 ± 3.93
6-12 hours	6.73 ± 3.68	21.0 ± 3.68
12 hours- 1 day	10.66 ± 3.53	18.3 ± 11.13
1- 2 days	18.3 ± 1.44	12.0 ± 1.44
2- 3 days	4.3 ± 0.33	1.66 ± 0.33
3- 4 days	1.1 ± 0.45	1.66 ± 0.45
4- 5 days	2.0 ± 0.57	1.0 ± 0.57
5- 6 days	2.3 ± 0.67	0.86 ± 0.67

All results represent the mean from three animals and the standard error unless otherwise noted.

*The mean of results from two animals.

radioactive material in the urine during the first 2 days. The results of I^{131} determinations on the liver, spleen, lungs, blood, and urine are shown in Table III and Fig. 4. The average 4-day antibody titers are shown in Fig. 2.

Thirteen depleted and thirteen nondepleted animals with 3 controls per diet group were used in the third experiment, which followed the general pattern of the first two. It differed only in that labeled sheep erythrocyte stromata were used as antigen in place of *S. typhi* vaccine. This variation was introduced to determine whether another labeled antigen, besides *S. typhi,* would

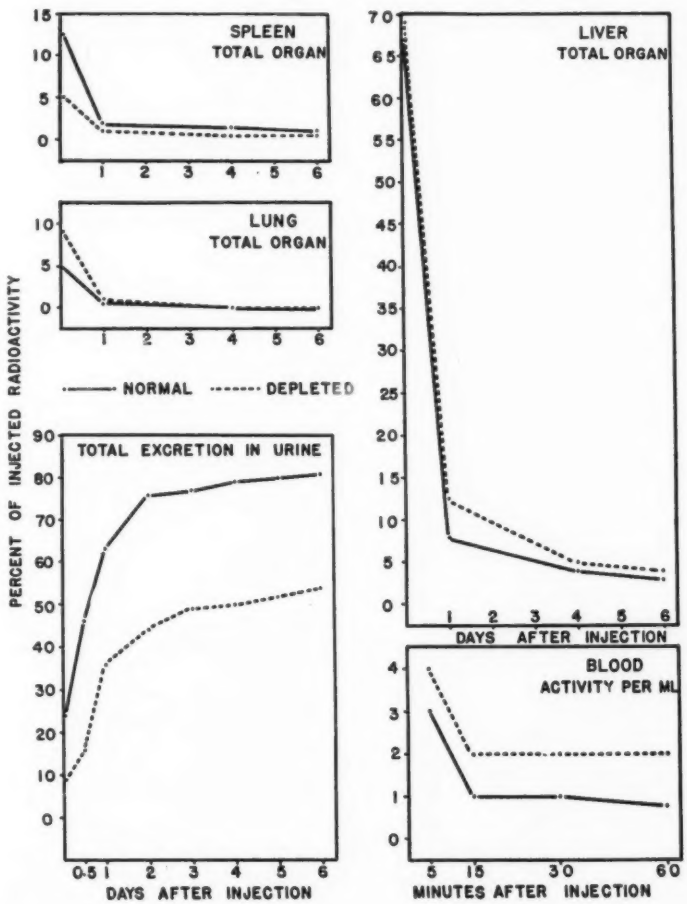


Fig. 4.—Experiment II. Radioactivity in various organs, urine, and blood following a single intravenous dose of I^{131}-labeled *S. typhi* vaccine in protein-depleted and control animals.

be distributed and digested similarly and whether it would lead to the same type of histologic reaction in the spleen. The measurements of the I^{131} content of the various organs and body fluids are shown in Table IV and Fig. 5. The greater localization of antigen in the spleens of the control rats was again observed a short time after antigen injection, confirming the difference observed in the first two experiments. The livers of depleted animals, which in

TABLE IV. DISTRIBUTION OF RADIOACTIVITY AND EXCRETION IN NORMAL AND DEPLETED ANIMALS FOLLOWING INJECTION OF LABELED SHEEP ERYTHROCYTE STROMATA

	PER CENT PER GRAM OF TISSUE					
	DEPLETED ANIMALS			NORMAL ANIMALS		
TIME	LIVER	SPLEEN	LUNG	LIVER	SPLEEN	LUNG
60 min.	2.46 ± 0.26	3.9 ± 0.47	4.9 ± 0.67	1.8 ± 0.0	5.0 ± 1.64	4.7 ± 1.34
8 hours	0.85 ± 0.22*	0.7 ± 0.0*	2.0 ± 1.00*	0.53 ± 0.17	0.7 ± 0.12	1.2 ± 0.38
1 day	0.4 ± 0.10	0.2 ± 0.04	0.2 ± 0.08	0.13 ± 0.04	0.2 ± 0.05	0.07 ± 0.02
4 days	0.09 ± 0.0†	0.06 ± 0.01†	0.02 ± 0.008†	0.07 ± 0.009	0.07 ± 0.01†	0.03 ± 0.02†
6 days	0.05 ± 0.0	0.04 ± 0.01	0.02 ± 0.014	0.03 ± 0.0	0.02 ± 0.02	0.01 ± 0.01

	PER CENT URINARY EXCRETION	
TIME	DEPLETED	NORMAL
0- 6 hours	4.8 ± 0.96	18.0 ± 3.60
6-18 hours	10.9 ± 7.00	8.4 ± 0.45
18 hours- 1 day	6.4 ± 1.64	7.4 ± 1.51
1- 2 days	19.0 ± 2.19	11.4 ± 3.24
2- 3 days	8.1 ± 1.30	4.5 ± 1.04
3- 4 days	2.1 ± 1.41	0.6 ± 0.17
4- 5 days	2.2 ± 0.52	0.5 ± 0.26
5- 6 days	3.0 ± 0.92	0.8 ± 0.26

All results represent the mean from three animals and the standard error unless otherwise noted.

*The mean of results from two animals.

†The mean of results from four animals.

the first 2 experiments had a higher I^{131} content compared with the livers of the controls, in this experiment contained less at first and then more than the controls at one day after injection. Furthermore, the total content of radioactivity in the livers of either the normal or the protein-depleted animals never became as great as was the case in previous experiments. This was also true, although to a lesser degree, in all the other organs. Urinary excretion of radioactive material, which was consistently lower in the protein-depleted rats in the first two experiments, was practically equal in the two dietary groups in this experiment.

In the first two experiments, the spleen of normal animals showed a significant increase in weight by the fourth day, which correlated with the rise in antibody titers and the cellular proliferation observed in the red pulp (Fig. 2). In the depleted animals, the weight increase was practically negligible. A similar result was obtained with relatively small doses of sheep red cells. Very large doses of stromata of labeled red cells also produced a definite increase in spleen weight as was observed in experiment IV (Fig. 2). There was little change in spleen weight in either dietary group when smaller quantities of labeled sheep red cell stromata were injected as in experiment III (Fig. 2).

Histology: Radioautographs: The radioactive material, as already described by Fitch and co-workers,[16] appeared to be localized in the marginal zone of the red pulp surrounding the Malpighian corpuscle and was interspersed focally in the rest of the red pulp. No I^{131} could be demonstrated in the white pulp (Fig. 6). In the liver, the distribution was focal and without any clear-cut lobular pattern. The sections of lung studied in the first and third experiments revealed localization of radioactivity over the lumen of the blood vessels, confirming the embolic nature of the high concentration of I^{131} in this organ

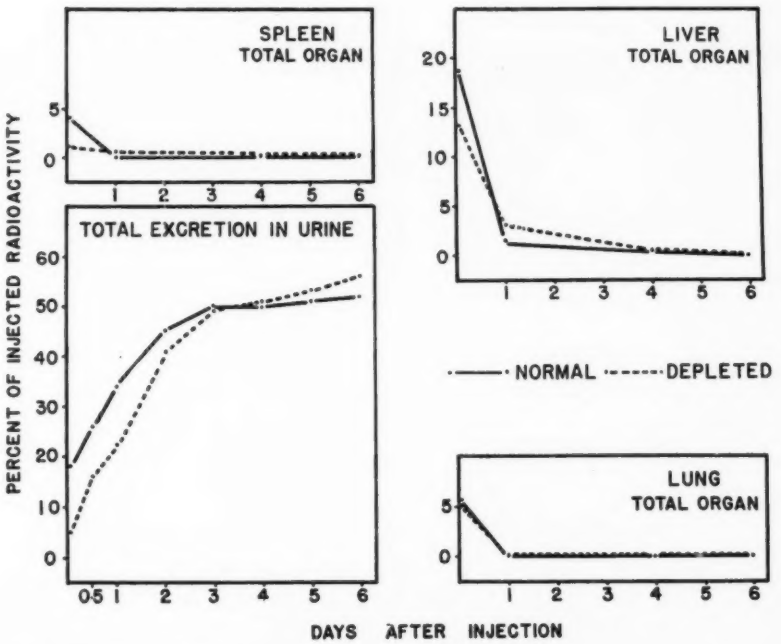


Fig. 5.—Experiment III. Radioactivity in various organs, urine, and blood following a single intravenous dose of I^{131}–labeled sheep erythrocyte stromata in protein-depleted and control animals.

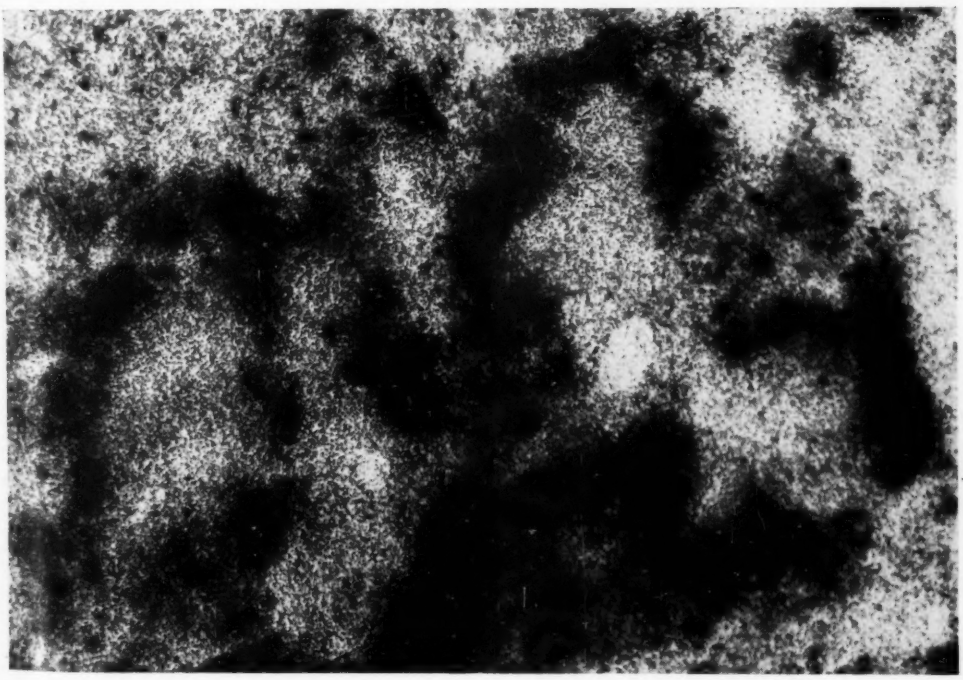
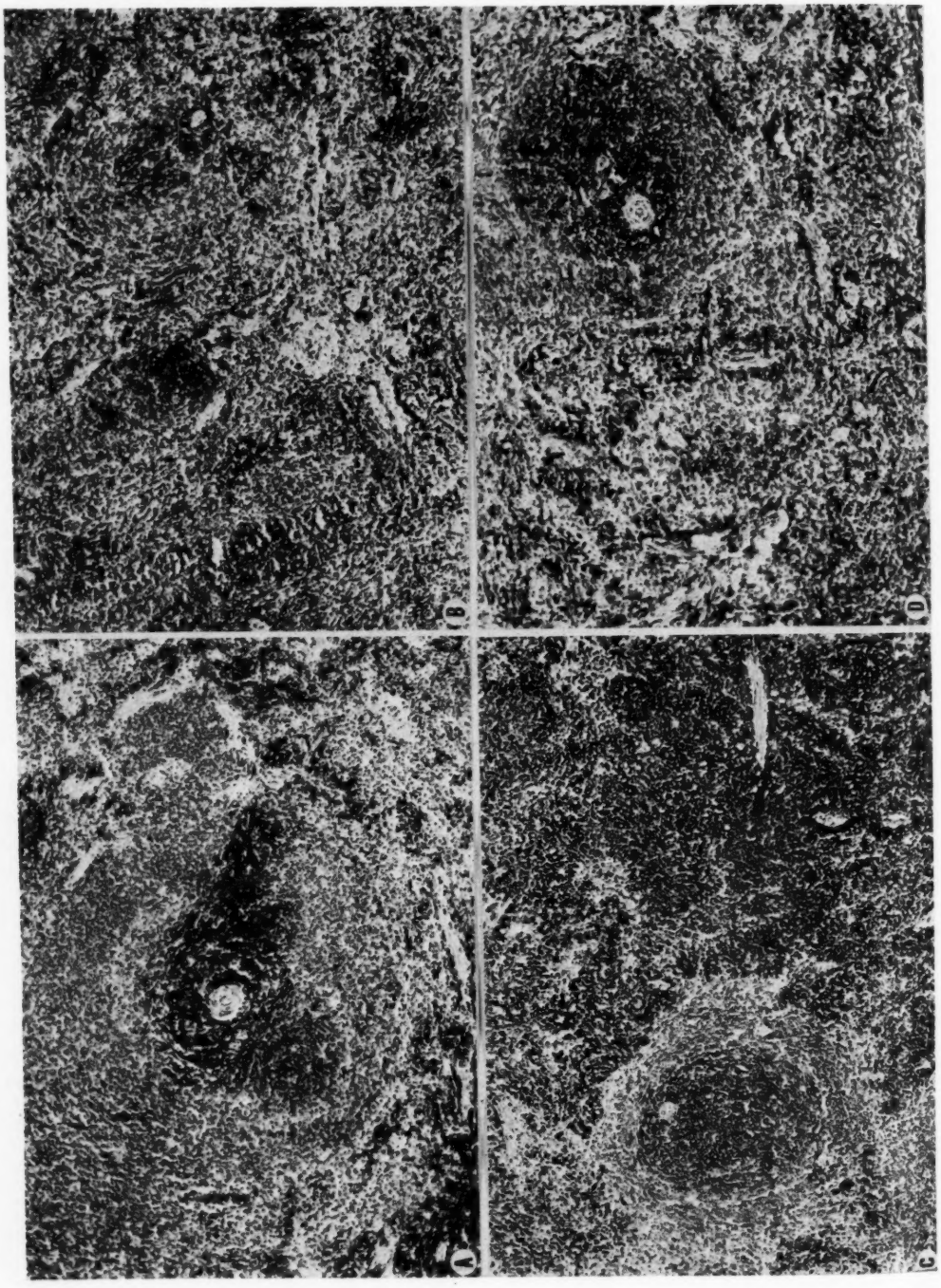


Fig. 6.—Distribution of I^{131}–labeled antigen in the spleen of a normal rat 15 minutes after injection. (Hematoxylin and eosin counterstain.)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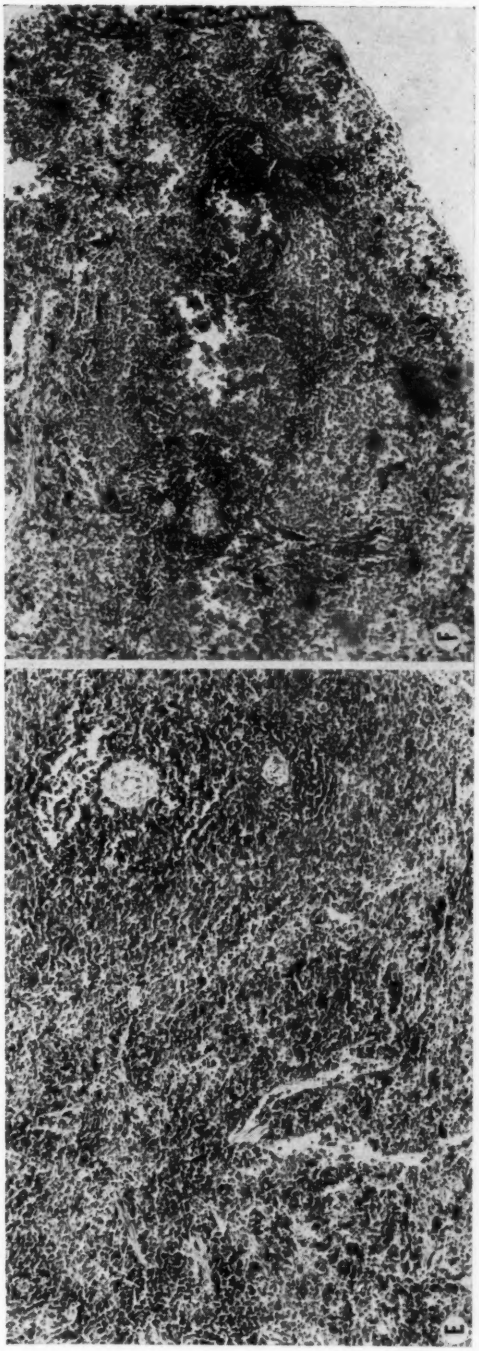

Fig. 7.—Spleens of protein-depleted and control rats at various intervals after labeled *S. typhi* vaccine injection. (Methyl green–pyronine stain.) *A*, normal, and *B*, depleted rats 60 minutes after injection; *C*, normal, and *D*, depleted rats 4 days after injection; *E*, normal, and *F*, depleted rats 6 days after injection.

early after injection (Fig. 3). The deposition of particles of India ink injected with the antigen in the second experiment, followed the same pattern of distribution as the radioactive antigen. This was demonstrated by the localization of the India ink in macrophages superimposed on the areas of tracks of radioactivity on the photographic emulsion.

Methyl green–pyronine stains: Spleens from normal animals at 15 and 60 minutes after antigen injection showed essentially a normal appearance with the usual small clumps of pyronine-positive cells in the red pulp and relatively inactive germinal centers in the Malpighian corpuscles. At one day after injection, the marginal zone was wider, the germinal center more active, and there was a slight increase in pyronine-positive cells in the red pulp in the typhoid vaccine-injected rats. There was little if any change at this time in the spleens of the control rats that had received stromata. By the fourth day after injection, the marginal zone was much narrower than on day 1 and the germinal center was very active and contained a large proportion of immature lymphocytes. The red pulp showed a maximum proliferation of immature pyroninophilic cells with many mitotic figures. These changes, although less pronounced, were still visible on the sixth day after injection (Fig. 7).

Cross sections of the spleen taken from protein-depleted animals 15 and 60 minutes after antigen injection were always smaller in size than those from spleens of normal animals. The Malpighian follicles were moderately to severely depleted of small lymphocytes. The marginal zones were less distinct and more diffuse although they did not vary much in size from those of nondepleted animals. Hemosiderin deposits in the red pulp were much more apparent than in normal animals. At one day after injection, the marginal zones were a little wider and the cross sections of the spleens appeared larger. By the fourth day, there was evidence of a variable but slight cellular proliferation, with the appearance of very small clumps of immature pyronine-positive cells in the red pulp and a narrowing of the marginal zones; mitotic figures were rare (Fig. 7).

In the third experiment, the changes seen in and around the Malpighian follicle of normal animals had characteristics similar to those seen in the previous ones. In the red pulp, however, the proliferation was less intense in the control animals and the difference in the reaction between the two groups was less apparent. Therefore direct determination of the per cent of pyroninophilic cells was undertaken. In each spleen, 500 to 700 cells in the red pulp were counted under oil immersion in several fields selected at random, and the percentage of pyronine-positive cells was calculated (Table V). The higher proportion of pyronine-positive cells in the spleens of nondepleted animals correlated with a higher titer of circulating antibody. The decreased cellular proliferation and the lower antibody titers observed when a standard intravenous dose of stromata was injected probably can be explained by a partial inactivation of the antigenicity of the iodinated stromata. The spleens from normal animals that had received an intravenous dose of red cell stromata 100 times larger than the standard immunizing dose of red cells used in this labora-

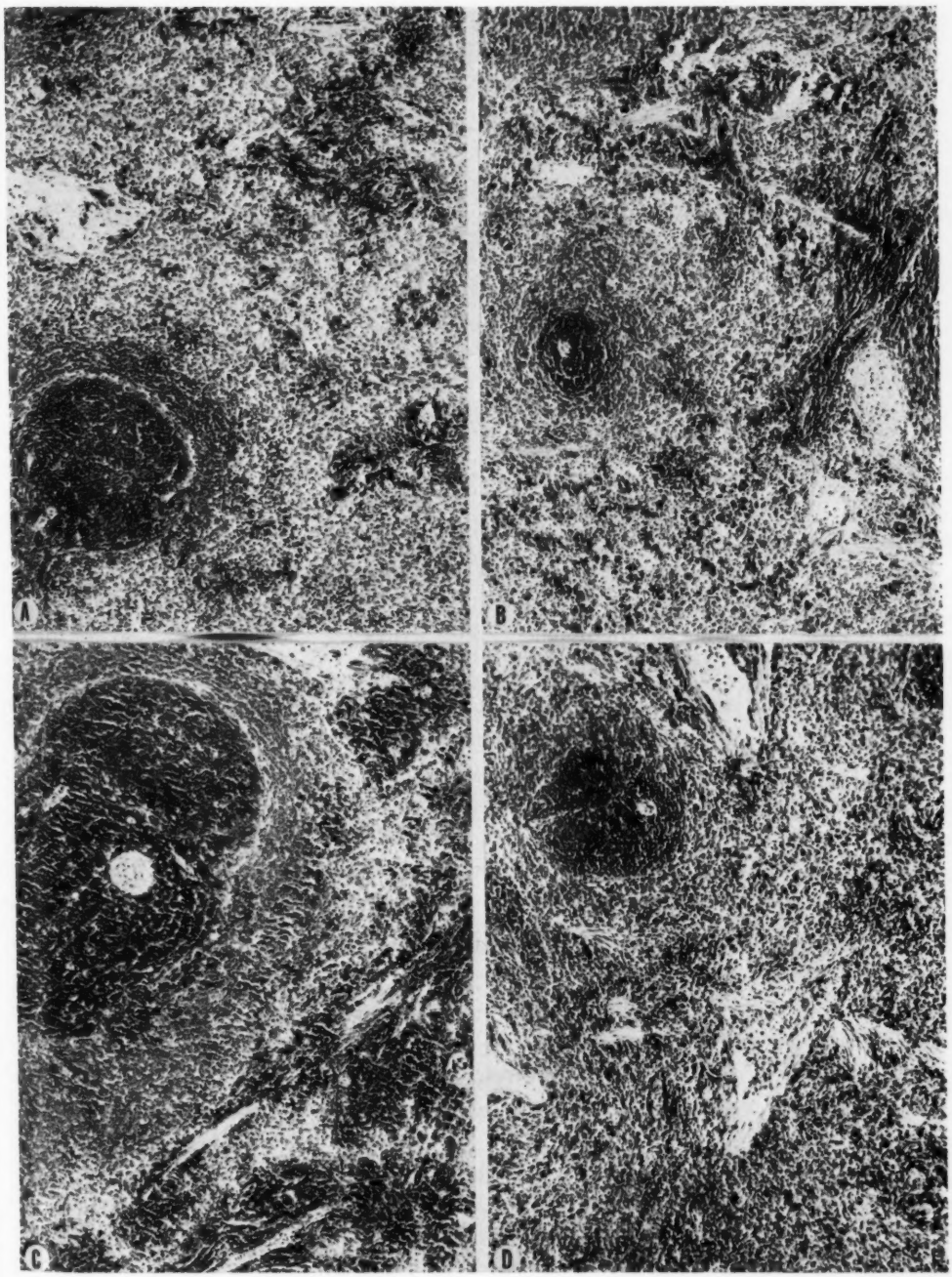

Fig. 8.—Spleens of protein-depleted and control rats at various intervals after a large dose of labeled sheep erythrocyte stromata. (Methyl green–pyronine stain.) *A*, normal, and *B*, protein-depleted rats 60 minutes after injection; *C*, normal, and *D*, protein-depleted rats 4 days after injection.

tory were histologically similar to those of nondepleted animals that received *S. typhi* vaccine or a standard dose of red cells (experiment IV). With this high dose of antigen given to the depleted rats, the proliferation observed was depressed as severely as in the protein-deficient rats injected with bacterial vaccine. Fig. 8 demonstrates the reaction of the splenic red pulp to this dose of stromata.

TABLE V. PER CENT OF PYRONINOPHILIC CELLS IN THE SPLEENS OF PROTEIN-DEPLETED AND CONTROL ANIMALS IN EXPERIMENT III

TIME AFTER INJECTION	DEPLETED	NORMAL
60 min.	1.2	3.8
8 hours	2.4*	1.9
1 day	1.3	1.6
4 days	3.1†	9.4†
6 days	1.8	8.5

All results represent the mean from three animals unless otherwise noted.
*The mean from two animals.
†The mean from four animals.

Extramedullary erythropoiesis complicated the interpretation of the cellular reaction in the red pulp of the spleen in the experiments reported by Fitch and co-workers.[16] This phenomenon was virtually absent in the spleens of rats used in experiments II, III, and IV because the animals that were bled to study the early peripheral blood clearance of antigen were not used for histologic studies.

In the second experiment, the localization of carbon particles in the spleens of both groups of animals was studied in relation to pyroninophilic proliferation. The carbon particles were localized initially in macrophages within the marginal zone and focally in the remaining red pulp. At 4 and 6 days, the majority of the macrophages containing carbon particles in the marginal zone appeared to have migrated away from the follicle and were surrounded by groups of large, intensely pyronine-positive, immature cells. No carbon could be seen inside these proliferating cells.

The spleens of noninjected animals from each dietary group appeared similar to those of injected animals from the same dietary group 15 minutes after antigen injection.

No change of significance was seen at any time after antigen injection in any of the remaining organs that were taken for histologic examination.

DISCUSSION

The stability of the I^{131} label on *S. typhi* has been investigated and described by Fitch and co-workers.[16] All tests of stability were repeated with the labeled sheep cell stromata, and the results showed that the I^{131}-stromata combination is stable in vitro. Since attempts to iodinate intact red cells proved unsuccessful and the antigenicity of stromata has been shown to be similar to that of intact cells,[17] I^{131} stromata were judged suitable for use as an antigen in the third and fourth experiments.

Most previous investigations of the relationship between cellular proliferation and antibody formation have utilized bacterial antigens, particularly from the enteric group. The endotoxin of *S. typhi* produces lymphorrhexis and might stimulate cell proliferation unrelated to antibody formation. Therefore, the histologic response to sheep erythrocyte stromata, a relatively nontoxic antigen, has been studied. It is apparent that the intravenous injection of sheep erythrocyte stromata in amounts equal to the standard dose of sheep red cells used in this laboratory is capable of producing proliferation of pyronine-positive cells of about the same degree observed after a standard dose of *S. typhi* vaccine. Since the cellular reaction has been elicited by this relatively nontoxic antigen, it seems probable that it is related to antibody formation and not just a reaction to endotoxin. This proliferation, observed in the spleens of immunized animals, has been linked by several authors to the content of ribose nucleic acid in the cells, and in turn to its rate of synthesis of protein—in this case antibody gamma globulin.[23-26] This appears to be true also in the experimental results reported here. It is evident that in protein-deficient animals, in which the histologic reaction is diminished or absent, the production of antibody is lower or lacking completely. When the animals were injected with different doses of stromata (experiments III and IV), the difference in the degree of histologic reaction was correlated with the difference in titer of circulating antibody.

Fitch and associates[27] have recently offered further evidence in support of the hypothesis of the interrelation between cellular proliferation and antibody formation. When the process was followed at 24-hour intervals, the correlation appeared to be one of dependence: at each interval the titer of circulating antibody reflected the cellular reaction 48 hours previously. When the animal was subjected to total-body x-irradiation a short time before antigen injection, the cellular reaction did not occur and no antibody was formed.[16, 27]

The apparent migration of macrophages containing intravenously injected carbon particles offers further evidence of the importance of the pyronine-positive cell in the production of antibody gamma globulin. These macrophages appear to migrate slowly from the marginal zone of the Malpighian corpuscle where most of the antigen was found very soon after injection. At 4 days, the majority of the phagocytosed carbon particles were located outside the marginal zone in the midst of clumps of pyroninophilic cells. The radioautographs indicated that carbon particles localized in the same cells as labeled particulate antigen. These observations may help to explain how the majority of the antigen, which initially was deposited in the marginal zone, is later moved into close proximity to the antibody-forming cells. There is good evidence on the important role that these pyronine-positive cells play in the fabrication of antibody. What remains to be answered is the question of their origin and their ultimate fate. They proliferate in large numbers between 1 and 4 days after antigen injection. By the sixth day, the cells vanish almost completely, leaving little trace of their existence, and the spleen

weight is reduced to almost normal values. There is no nuclear debris or any other sign of cell death. A few cells may develop into mature plasma cells, but on the whole they cannot be accounted for.

As observed by others[28] protein depletion depresses the rate of phagocytosis by the reticuloendothelial system of the spleen. This phenomenon is confirmed by the present study.

Cannon and associates[1-3, 9] and others[4-8, 10] established the close relation existing between the state of the protein reserves and the formation of antibody. The results of the present work help elucidate the cellular mechanism of this depression of antibody synthesis. There are some differences between this and other instances in which the immune response is inhibited. In contrast to the action of total-body x-irradiation, which seems to spare the phagocytic activity of the reticuloendothelial system, protein depletion seems to depress the phagocytic activity of the spleen. Whether this is of primary importance as far as the synthesis of antibody is concerned is not yet completely clear. Cortisone does not apparently influence antigen uptake or antibody release but seems to interfere specifically with the synthesis of antibody protein, probably at the cellular level.[29] Wissler and associates[30] have recently studied the acute effects of feeding certain amino acid analogues on the formation of antibody and the cellular proliferation in the spleen. Both of these phenomena were inhibited when the analogue was substituted for one of the essential amino acids.

In conclusion it may be said that although prolonged protein deficiency may reduce resistance to infection in many ways,[4, 5, 31] in the present study the deficiency seemed to affect the immune mechanism of the rat mainly by: (1) reducing the phagocytic rate and the total phagocytic capacity of the macrophages of the spleen; (2) depressing the proliferation of pyroninophilic cells in the red pulp of the spleen following a single intravenous dose of a particulate antigen; (3) lowering the humoral antibody concentration in response to a single injection of antigen given intravenously.

The cellular proliferation, described by several authors as being linked intimately to the formation of antibody, has been elicited in the spleen of the normal rat in response to an intravenous injection of antigens other than bacteria of the enteric type. This observation provides further evidence of the importance of the role of cellular hyperplasia and transformation which accompany antibody synthesis.

SUMMARY

Adult male albino rats were kept on a protein-deficient diet until they lost between 25 per cent and 40 per cent of their body weight. At the end of this period, depleted animals and controls were injected with a dose of I^{131}-labeled *S. typhi* or sheep erythrocyte stromata. Blood clearance, tissue distribution, and excretion of radioactive material were studied. The cellular localization and the histologic reaction to the stimulus were followed at suitable intervals. The rate of phagocytosis in the spleen was lower for one day post-injection in the depleted animals, whereas the rate of disappearance from other organs was usually similar

in the two groups. Radioactive material was localized focally in the red pulp of the spleen and in the marginal zone around the Malpighian follicle. The cellular response of the spleen consisted of hyperplasia of the red pulp which was accounted for mainly by an increase in pyronine-staining cells. This response was decreased in the protein-depleted animals.

The relation of these phenomena to reduced antibody synthesis following protein depletion is discussed.

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A FURTHER EVALUATION OF HISTOPLASMIN AND YEAST PHASE ANTIGEN OF HISTOPLASMA CAPSULATUM IN THE COMPLEMENT FIXATION TEST

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COMPLEMENT fixation tests for the detection of infection with *Histoplasma capsulatum* are becoming increasingly useful in the diagnosis of this disease. In some laboratories these tests are performed exclusively with histoplasmin (the mycelial filtrate or skin test antigen), while in others they are carried out with antigens from the yeast or parasitic phase of the organism. Although there are several published reports relating to serologic data obtained with one of these two types of antigen,[1-5] the literature reveals only one study in which histoplasmin and yeast phase antigens were used in simultaneous complement fixation (CF) tests.[6] The latter report described serologic findings in 26 human cases of histoplasmosis, 11 of which were verified by isolation of the organism, while 16 were presumptively diagnosed by clinical symptoms, pulmonary lesions, and conversion of histoplasmin skin test from negative to positive. Sera from the verified cases fixed complement in the presence of histoplasmin to titers ranging from 1:32 to 1:16,384, but in every instance either failed to fix complement with yeast phase antigen or reacted with it only in low dilution (0 to 1:32). These findings, which suggest that histoplasmin is the superior antigen for demonstrating CF antibody in suspected or proved cases of histoplasmosis, are at variance with results obtained in this laboratory where histoplasmin and whole yeast phase antigens also are used in simultaneous complement fixation tests.

The purpose of this report is to describe results that were obtained with the two types of antigen in sera from 13 culturally verified and 96 presumptive cases of histoplasmosis; to point out that while there is considerable variation in the reactivity of the two antigens, the higher, and often significantly higher, reactions are not consistently associated with either type; to demonstrate that sera from some cases fix complement in high titer with one type of antigen while failing to react even in lowest dilution with the other; and, finally, to emphasize the importance of employing both types of antigen in all suspected cases of this disease. In order to minimize the possibility of selecting sera from certain types of cases of histoplasmosis for evaluation, the data presented were drawn from more than 1,000 consecutive sera that were received in this laboratory for CF tests for histoplasmosis, blastomycosis, and

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coccidioidomycosis over a period of approximately 5 months. To circumvent, at least in part, the problem of cross reactions in mycotic antigens, only those specimens with complement fixation titers of 1:32 or above are discussed.*

MATERIALS AND METHODS

A. *Antigens.*—

1. The histoplasmin used in this study was prepared and generously supplied by Parke, Davis & Company. This preparation (Lot H-15) was a filtrate of liquid synthetic media[7] in which the mycelial phase of Emmons' Strain 6,510 of *Histoplasma capsulatum* was cultured over a period of approximately 3 months. The filtrate was used at a dilution of 1:4. At this dilution, no anticomplementary action was noted and maximum titers were demonstrated in sera from 10 culturally verified cases of human histoplasmosis. The dilutions of antigen in these preliminary titrations ranged from 1:2 to 1:512.

2. The whole yeast phase antigen (WYP) was prepared in this laboratory. Mass quantities of Strain G-8 of *H. capsulatum* (a human isolate) were grown on brain-heart infusion agar (DIFCO) for 48 hours at 37° C. The organisms were harvested and killed in physiologic saline solution containing 0.5 per cent formalin, thoroughly washed in physiologic saline solution, and inactivated for 2 hours at 60° C. Finally, a stock suspension was prepared in a Veronal bicarbonate buffered salt solution (pH 7.4) and preserved with Merthiolate at a final concentration of 1:10,000. The stock suspension, maintained at 4° C., was diluted 1:100 for use in the tests, as it was with this concentration of antigen that maximum titers were obtained in preliminary titrations with sera from 10 culturally verified cases of histoplasmosis in humans. At the latter dilution, the antigen contained 0.45 ml. packed yeast cells in 100 ml. diluent, following centrifugation at 2,500 r.p.m. for 5 minutes.

B. *Complement Fixation Procedure.*—As in similar studies from this laboratory,[2, 4] the method used was a development of earlier quantitative procedures:

The reagent diluent was a Veronal bicarbonate buffered salt solution (VBS) of pH 7.3-7.4 containing Mg^{++} and Ca^{++} in concentrations optimal for immune hemolysis.[8] Preserved sheep's blood served as source of erythrocytes.[9] Glycerolized hemolytic antiserum and desiccated guinea pig complement (C′) were obtained commercially. The standard volume for tests and titrations was 1.5 ml., 0.3 ml. aliquots being allotted the 5 reagents essential in complement fixation; adjustments to standard volume were made when necessary with VBS. The suspension of washed erythrocytes was adjusted spectrophotometrically to contain 5×10^8 cells per milliliter, and sensitized with an optional concentration of hemolysin. The 50 per cent hemolytic unit (C'_{H50}) of complement was determined by a precise spectrophotometric technique.[10]

In the CF tests performed in this laboratory, the serum, complement ($5C'_{H50}$), and antigen were incubated for 16 to 18 hours at 3 to 6° C. for fixation and for an additional period of 30 minutes at 37° C. following the addition of the hemolytic system. Tubes containing unlysed cells were centrifuged at 2,500 r.p.m. for 5 minutes, after which the percentage of hemolysis in the supernate was visually compared with simulated quantitative standards. Serum dilutions in which there was 0 to 50 per cent hemolysis were considered positive; titer was expressed as the reciprocal of the highest dilution of serum yielding a positive reaction.

RESULTS

As outlined in Table I, 292 (27.3 per cent) of the 1,074 sera in this series fixed complement to a titer of 1:8 or above with either histoplasmin or the whole yeast phase antigen.* However, of the latter specimens, only 146 (50 per cent) reacted to titers as high as 1:32 or more, although in approximately

*All sera in this study are from cases which, as far as can be ascertained, have not been described in any other published reports.

⅔ of the sera, the higher titers were not obtained with the same antigen. There were, for example, 57 specimens which reacted to higher CF titer with the whole yeast phase antigen and 41 sera in which histoplasmin was the more highly reactive. Only 48 sera, or approximately ⅓ of the "positive" (CF levels of 1:32 or above) specimens exhibited essentially similar titers with both antigens.

TABLE I. SUMMARY OF CF RESULTS WITH HISTOPLASMIN AND WHOLE YEAST PHASE ANTIGEN (WYP) OF HISTOPLASMA CAPSULATUM IN 1,074 SERA SUBMITTED FROM SUSPECTED CASES OF HISTOPLASMOSIS, BLASTOMYCOSIS, AND COCCIDIOIDOMYCOSIS

	NO.	PER CENT
A. All specimens:		
1. Negative titers—both antigens	663	61.7
2. Titer of 1:8 or above—one or both antigens	292	27.3
3. Anticomplementary specimens	119	11.0
4. Total specimens	1,074	100.0
B. Specimens reacting with histoplasma antigens:		
1. Titers of 1:8 or 1:16—one or both antigens	146	50
2. Titers of 1:32 or above—one or both antigens	146	50
3. Total specimens	292	100
C. Specimens with CF titers at 1:32 or above:		
1. Essentially equal—both antigens	48	32.87
2. Higher titer with WYP antigen	57	39.04
3. Higher titer with histoplasmin	41	28.09
4. Total	146	100.0

TABLE II. COMPARISON OF CF TITERS IN 41 SERA (33 CASES) IN WHICH HISTOPLASMIN REACTED TO HIGHER TITER THAN WHOLE YEAST PHASE ANTIGEN (WYP)

NO. SERA	CF TITER WITH WYP ANTIGEN	NO. SERA WITH CF TITER TO HISTOPLASMIN AT:			
		1:32	1:64	1:128	1:256
30	<1:8	14	10	4	2
5	1:8	4		1	
5	1:16		5		
1	1:32				1

TABLE III. COMPARISON OF CF TITERS IN 57 SERA (32 CASES) IN WHICH WHOLE YEAST PHASE ANTIGEN (WYP) REACTED TO HIGHER TITER THAN HISTOPLASMIN

NO. SERA	CF TITER WITH HISTOPLASMIN	NO. SERA WITH CF TITER TO WYP ANTIGEN AT:				
		1:32	1:64	1:128	1:256	1:512
34	<1:4	19	8	4	1	2
5	1:8	2		1	2	
11	1:16		3	3	3	2
6	1:32			5	1	
1	1:64					1

TABLE IV. COMPARISON OF CF TITERS IN 48 SERA (34 CASES) IN WHICH REACTIONS WITH HISTOPLASMIN AND WHOLE YEAST PHASE (WYP) ANTIGEN WERE ESSENTIALLY EQUAL

NO. SERA	CF TITER WITH WYP ANTIGEN	NO. SERA WITH CF TITER TO HISTOPLASMIN AT:				
		1:16	1:32	1:64	1:128	1:256
7	1:16		7			
23	1:32	5	10	8		
14	1:64		4	7	3	
2	1:128			1		1
2	1:256				1	1

TABLE V. SUMMARY OF FINDINGS IN 13 CULTURALLY VERIFIED CASES OF HISTOPLASMOSIS

CASE NO.	PERTINENT DATA	TIME AFTER ONSET	CF TITER		RESIDENCE
			WYP	HISTOPLASMIN	
1	63-year-old white, male farmer. *H. capsulatum* isolated repeatedly from sputum. Chest x-ray reveals extensive bilateral disease with cavity in right apex. Histoplasmin skin test 3+.	12 months 14 months 18 months	1:128 1:128 1:128	1:64 1:32 1:32	Tennessee and Missouri
2	Adult man. *H. capsulatum* isolated from sputum.	12 months	1:16	1:32	Virginia
3	62-year-old man. *H. capsulatum* recently isolated from sputum. History of "lung trouble" for 20 years. Chest x-ray reveals moderately advanced bilateral disease consistent with pulmonary tuberculosis. Cavity 3 × 3.5 cm. LUL. Skin test reaction to PPD 3+, histoplasmin +. Cultures for AFB negative.	1 or 20 years	1:64	1:64	Kansas and Missouri
4	60-year-old white man. *H. capsulatum* isolated within past year from sputum. Chest x-ray reveals pulmonary fibrosis and collapse of left lung. Chest pathology known for 37 years. Acute episode 7 years ago. Chronically ill since.	7 or 37 years. 1 month later 4 mos. later 6 mos. later	1:32 1:32 1:16 1:16	1:64 1:64 1:64 1:64	Tennessee
5	27-year-old white man with illness of more than 1 year. Splenectomy 1 year ago for thrombocytopenic purpura. Bone marrow reticuloendothelial cells contained round oval bodies typical of *H. capsulatum*.	>1 year	1:64	1:64	Canal Zone
6	54-year-old white man. *H. capsulatum* isolated 1 year ago from sputum. Pulmonary inflammatory lesion in left upper chest of 2 years' duration. Skin test reactions to PPD and histoplasmin positive.	>1 year	1:64	1:64	Tennessee
7	37-year-old white man. *H. capsulatum* isolated from sputum. Skin test reactions positive to histoplasmin and PPD. Chest x-ray reveals cavity in LUL.	2 years	1:16	1:32	Tennessee
8	Adult man. *H. capsulatum* isolated from node biopsy. History of recurrent pneumonitis on 6 occasions. Latest episode 6 weeks ago. Chest symptoms for 10 years. X-rays reveal infiltration both lungs and bilateral cavitation.	10 years	1:32	1:256	Michigan
9	42-year-old white man with bilateral apical infiltration and progressive linear nodular infiltrates persisting for 1 year after onset of acute pneumonitis. *H. capsulatum* isolated from sputum. Skin tests positive with histoplasmin and PPD. Patient is a laboratory technician with constant exposure to TBC.	1 year 14 months 18 months	0 0 0	1:64 1:128 1:128+	Pennsylvania and New York

10	58-year-old white man. *H. capsulatum* isolated from sputum on 2 occasions. Cerebral thrombosis. Low grade fever. Chest x-ray reveals infiltration both apices with suggestion of cavitation.	6 months to 1 year	0	1:64	Louisiana
11	63-year-old white man with sessile, polypoid lesions in both tonsils and rectum. Diarrhea of many years' duration. Chest x-ray discloses soft pulmonary infiltrations. *H. capsulatum* isolated from polypoid lesion in rectum.	?	1:32	0	Virginia
12	28-year-old white man with acute pneumonitis. Chest x-ray disclosed diffuse infiltration and "dense, miliary disease, grossly nodular." *H. capsulatum* isolated from sputum, gastric washings, and soil with which patient was working 2 weeks prior to onset.	2 weeks 3 weeks 6 weeks	1:512 1:256 1:128	1:64 1:32 0	Delaware
13	78-year-old white man with history of chronic lung disease. Chest x-ray shows infiltration in RUL. *H. capsulatum* isolated from sputum.	?	1:32	0	Tennessee

TABLE VI. SUMMARY OF FINDINGS IN 5 PRESUMPTIVE CASES OF HISTOPLASMOSIS WHEREIN CF TITERS WERE SIGNIFICANTLY HIGHER WITH THE WYP ANTIGEN

CASE NO.	PERTINENT DATA	TIME AFTER ONSET	CF TITER		RESIDENCE
			WYP	HISTOPLASMIN	
14	40-year-old man with dyspnea, fatigue, and occasional spiking of fever for past 2 years. Chest x-ray currently reveals bilateral lung infiltrate. Splenomegaly.	24 months 27 months	1:256 1:64	0 0	Michigan
15	18-year-old white man with bilateral hilar lymphadenopathy. Considerable clearing of infiltration in right mid-lung field 3 months after onset.	>3 months >5 months >7 months	1:128 1:64 1:32	1:16 1:16 1:16	
16	24-year-old white man with acute, febrile illness two weeks after cleaning house. History of employment for 6 months on chicken farm terminating just prior to episode. Chest x-ray revealed diffuse, miliary nodulations in both lung fields and right hilar lymphadenopathy. Skin test with histoplasmin converted from 1+ to 3+ during hospitalization.	1 year (?) 14 months 18 months	1:512 1:256 1:128	1:16 1:8 0	Ohio Tennessee Arkansas
17	25-year-old white man who 5 weeks prior to admission developed "summer pneumonia." Chest x-ray revealed numerous nodular densities in both lung fields in contrast to negative earlier film. History of recent vacation in rural Indiana and exposure to dust storm in Kentucky en route to home in Alabama.	6 weeks 10 weeks	1:512 1:128	1:16 0	Alabama
18	19-year-old white men with generalized pulmonary infiltration which subsided without treatment in 10 days. History of recent acute illness with dyspnea, cough, and malaise. Histoplasmin skin test converted from negative to positive during 6-week observation period, but old chest film showing calcified lung lesions suggests that this is recurrent infection.	3 months 6 months	1:32 1:64	0 0	Canal Zone

TABLE VII. SUMMARY OF FINDINGS IN 5 PRESUMPTIVE CASES OF HISTOPLASMOSIS WHEREIN CF TITERS WERE SIGNIFICANTLY HIGHER WITH HISTOPLASMIN

CASE NO.	PERTINENT DATA	TIME AFTER ONSET	CF TITER		RESIDENCE
			WYP	HISTOPLASMIN	
19	Adult man with cough, nausea, and production of grayish sputum. Patient unwell for 10 years. Diagnosis 2 years ago of "spasmodic colon." Lost vision in left eye 5 years ago —sight has since returned. Currently brucellosis titer 1:500.	?	0	1:128	Texas
20	Adult man with dyspnea and cough for 1 year. Chest x-ray reveals pleural effusion on left with marked pleural thickening entire left cavity. Cultures for AFB negative. Skin test with histoplasmin positive, PPD negative.	1 year	0	1:256	Georgia and Florida
21	23-year-old man admitted to sick call because of massive pleural effusion. Chest x-ray currently reveals old, dried, adhesive, inactive pleural effusion. Skin tests with histoplasmin, coccidioidin, and PPD positive.	?	0	1:64	Massachusetts and New York
22	25-year-old colored man with history of hospitalization 4 years ago in California for suspected sarcoidosis. Readmitted to hospital 3 months ago with abdominal complaints, headache, and fever. Chest x-ray reveals multiple cavitations. Skin tests with histoplasmin and PPD positive, coccidioidin, negative.	?	0	1:32	California and Texas
23	44-year-old white man hospitalized 3 months ago because of LUL pneumonia which did not respond to treatment. Some clearing of lung infiltration during hospitalization. Skin tests with histoplasmin and PPD positive.	>3 months	1:16	1:64	West Virginia

Not only were differences in titer with the two types of antigen observed, but in many cases these differences were very wide. The data shown in Table II reveal that 30 sera which were essentially negative with the WYP antigen reacted with histoplasmin to CF titers which ranged from 1:32 to 1:256. A reversal of this activity, however, was also observed, and, as shown in Table III, 34 of 57 additional specimens reacted to CF levels of 1:32 to 1:512 with the WYP antigen but failed to fix complement even in lowest dilution with histoplasmin. In addition, there were 11 sera listed in Table II and 23 sera in Table III in which substantial differences in CF levels were also obtained with histoplasmin and the WYP antigen, respectively.

The 48 sera in which CF titers were essentially equal with both types of antigen are illustrated in Table IV. Such levels ranged from 1:16 to 1:256.

CF Findings in Culturally Verified Cases.—Persistent pulmonary lesions and a positive skin test reaction to histoplasmin were prominent findings in many of the cases with CF titers at 1:32 or above and the clinical diagnosis of histoplasmosis was confirmed in 13 patients by isolation of *H. capsulatum*. Pertinent data in the latter cases are summarized in Table V. It will be noted

that while sera from 7 of these persons reacted to similar titer with both types of antigen (Cases 1 to 7), there were 3 persons whose sera reacted at dilutions of 1:64 to 1:256 with histoplasmin but who showed minimal or negative titers with whole yeast phase antigen (Cases 8, 9, and 10). In Cases 11, 12, and 13, this order of reactivity was reversed, and it was with the WYP antigen that histoplasmosis antibody was demonstrated in significantly higher titers.

CF Titers in 10 Presumptive Cases.—The lack of correlation in the reactivity of the two types of antigen is demonstrated in Tables II, III, and V, and additional evidence of the marked differences in titers repeatedly observed in this study is presented in Tables VI and VII. *Histoplasma capsulatum* was not isolated from these 10 patients, but their clinical symptoms, chest pathology, and skin test reactions to histoplasmin were consistent with those found in many culturally verified cases. It will be seen that sera from the 5 cases described in Table VI reacted to CF titers that were considerably higher with the WYP antigen. In the 5 cases shown in Table VII, on the other hand, significantly higher titers were demonstrated in the presence of histoplasmin. The values shown in both tables reveal that in each instance, titers with the less reactive antigen not only were low, but frequently negative.

DISCUSSION

The data presented in this study were obtained from complement fixation titrations on more than 1,000 sera that were submitted for serologic tests for histoplasmosis, coccidioidomycosis and blastomycosis over a period of 5 months. In testing for *H. capsulatum* complement fixing antibody both histoplasmin and WYP antigen were employed in simultaneous tests. An analysis of the findings in 146 sera which fixed complement to a titer of 1:32 or above with one or both of these antigens disclosed a marked lack of correlation in the reactivity of the two types of preparation. In some cases, the significantly higher titer was observed with the WYP antigen, while in others histoplasmin was the more highly reactive antigenic preparation.

Histoplasmosis has long been noted for its extremely protean clinical manifestations and thus, as might be anticipated, comparable serologic reactions were often associated with cases showing extremely diverse symptomatology. Nevertheless, there were at least two relatively consistent findings. The first of these related to the WYP antigen which reacted to much higher titer than did histoplasmin in sera from early, primary, pulmonary histoplasmosis, or in cases with evidence of a recent recurrence of infection. This is illustrated in Case 12, Table V, where a CF titer of 1:512 was demonstrated with the WYP antigen as early as the second week after onset. The histoplasmin CF titer, on the other hand, not only did not exceed 1:64 at any time but reverted to negative within the first 6 weeks of observation. In this case, diagnosis of histoplasmosis was verified by isolation of the organism from the patient as well as from the contracting source in nature. Similar case histories were obtained in presumptive Cases 15, 17, and 18, Table VI, in each of which the WYP antigen was also the more highly reactive. The second consistent

finding concerned histoplasmin. This antigen appeared to be the more sensitive preparation for demonstrating antibody in cases of longer duration, especially those with circumscribed, single or multiple, pulmonary lesions. The presence of cavitation, fibrosis, or pleural effusion in persons with significant titers of CF antibody demonstrable with histoplasmin but not always with WYP antigen was not uncommon (Cases 8 and 10, Table V, and Cases 20, 21, and 22, Table VII).

Except for these two very general considerations, it is unwise to postulate which of the two antigens might provide more informative data under certain specified clinical conditions. Further, it is debatable as to which type of preparation has the greater specificity. Cross reactions occurred with blastomyces antigen in many of these sera and were as marked and as frequent in those reacting with histoplasmin as with the WYP antigen. One notable cross reaction also persisted in a series of sera from a case of human coccidioidomycosis with a coccidioidin CF titer of 1:1,024. In sera from this patient, histoplasmin as well as the WYP antigen repeatedly reacted to a CF titer of 1:32. It is also of interest to note that in 3 culturally verified cases of histoplasmosis not described in Table V, CF titers of 1:8 and 1:16 were obtained with both types of antigen in 2 and of 1:16 with the WYP antigen only in the third. Titers of this low magnitude were also observed with both antigens in 1 culturally verified case of cryptococcosis and 3 additional cases of coccidioidomycosis.[4] In view of these data, there is no alternative but to recommend the use of both types of antigen in all suspected cases of histoplasmosis.

This conclusion appears to be at variance with that reached by Schubert and his associates,[6] who reported CF titers of 1:32 to 1:16,384 with histoplasmin in sera which, with few exceptions, were negative with yeast phase antigens prepared from the same strain of *H. capsulatum*. While the high titers observed in many of their cases leave little doubt that these investigators employed a superior lot of histoplasmin, the almost total absence of reaction with the 2 yeast phase preparations tends to be misleading. The whole yeast cell type of preparation especially is used with considerable success by other investigators.[3-5] Its failure to react in the series presented by Schubert and associates might possibly be attributed to one of the following factors: (1) the inadvertent selection of sera comparable to those in Table II, all of which reacted to higher CF titer with a different and presumably less potent lot of histoplasmin than that used by Schubert, (2) the use of unsuitable preparations of yeast phase antigens, since these, like histoplasmin, vary in complement-fixing activity with mode of production, lot, and strain,[11-13] or (3) the use of adequate yeast phase antigens at other than their optimum dilutions, since, as noted in an earlier report, a serum suitable for determining the optimum activity of histoplasmin might not be equally suitable for determining that of a yeast phase antigen, and vice versa.[14]

Further corroboration of the usefulness of yeast phase antigens is provided in the present report, where such a preparation not only was reactive but in many instances reacted to a higher titer than histoplasmin. It would,

therefore, be unfortunate if the report by Schubert and his co-workers, which so dramatically demonstrated the superiority of histoplasmin in certain cases, were at the same time to discourage the use of yeast phase antigens. Until considerably more information relating to their antigenic composition is at hand, it is our opinion that neither of these preparations should be used to the exclusion of the other.

SUMMARY

Histoplasmin and a whole yeast phase (WYP) antigenic preparation of *H. capsulatum* were employed simultaneously in determining the level of CF antibody in more than 1,000 sera submitted for routine serologic tests for histoplasmosis, blastomycosis, and coccidioidomycosis. Evaluation of these data revealed that of 146 sera which reacted to CF levels of 1:32 or above with one or both antigens, 57 reacted to significantly higher titer with WYP antigen, 41 to significantly higher titer with histoplasmin, while only 48 reacted to essentially equal titers with both antigens. From these data it was concluded that both types of preparations should be employed in all suspected cases of histoplasmosis.

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CHLORPROMAZINE-INDUCED DIURESIS

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PREVIOUS reports[1, 2] have shown that the vascular effects of chlorpromazine* are hypotension and decreased peripheral resistance, with no effect on renal hemodynamics unless hypotension occurs.

Because of several unexpected responses to the drug in patients with uremia, a further study of renal function, especially during the first hour after administration, was undertaken.

METHODS

I. *Effects on Renal Functions.*—Renal functions were studied in 12 convalescent patients without clinical evidence of renal disease or hypertension, with use of inulin clearance to measure glomerular filtration (GFR) and sodium para-aminohippurate clearance to measure effective renal plasma flow (ERPF).[3, 4] Sodium and potassium were determined in both serum and urine by means of a Barclay flamephotometer. Values for renal functions were converted to a body surface area of 1.73 m^2. In 10 of the 12 patients studied, 3 control periods of urine collection were made, and in the remaining 2 patients only 2 periods were obtained. Twenty-five milligrams of chlorpromazine diluted to 10 ml. with 0.83 per cent saline was given intravenously slowly over a 4- to 5-minute period. During this time the blood pressure was checked at 30-second intervals by a standard sphygmomanometer attached to the opposite arm. Following the injection, urine was collected in 10-minute periods for an hour or more. Blood pressure was recorded at 5-minute intervals subsequent to the injection. At no time did any of the 12 patients included in the study have a fall in blood pressure.

In the calculation of the results, the 2 to 3 periods of urine collection before the injection of the drug served as the control for each patient. The variation from the control mean was determined, and it was assumed that had nothing further been done, this average variation would have been expected throughout the remainder of the experiment. A change from the control mean greater than this would be significant depending on statistical analysis. The variation from the control mean was averaged in all the patients, as was the change from the control mean for each successive period following the administration of the drug and the maximum change following the drug. The "t" value was then determined for the difference between the means and the statistical analysis expressed as "p." If the "p" value was greater than 0.05, it was not considered significant enough to record.

II. *Effect of Chlorpromazine on ADH Excretion.*—Urine collections were made from 7 to 9 A.M. and 9 to 11 A.M. in 20 normotensive convalescent patients. In 10 of these, after the first 2-hour urine sample was collected, 25 mg. of chlorpromazine was given slowly intravenously over a 4- to 5-minute period with use of the same precautions as before. None of the patients included in the study had a fall in blood pressure during the procedure.

The urine in each instance was extracted by the method of Grollman and Woods[5] and the final extract dissolved in distilled water so that each milliliter of extract was equivalent to 15 minutes of urine excretion. The extracts were then assayed in female rats, who

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simultaneously received water by stomach tube, equivalent to 5 per cent of their body weight, and 1 ml. of the final urine extract injected intraperitoneally. Six animals were used for each urine specimen (12 for each patient). The results were calculated as the per cent of the hydrating dose of water excreted in 120 minutes by the rats.

In comparing the effect on hydrated rats of the first and second 2-hour urine specimens in each patient, we assumed that the greater the urine excretion in the rats the less the ADH-like material there was present in the patient's urine sample. If the second 2-hour specimen resulted in a greater excretion of urine in the rats than the first 2-hour specimen, it was assumed that there had been a decrease in excretion of ADH-like material by the patient.

RESULTS

The effect of chlorpromazine on renal function in 12 patients is tabulated in Table I and illustrated in Fig. 1. During the control periods there was an average glomerular filtration rate of 114 ± 14 ml. per minute with an average variation from the control means in each individual of 8.5 ± 6.3 ml. per minute;

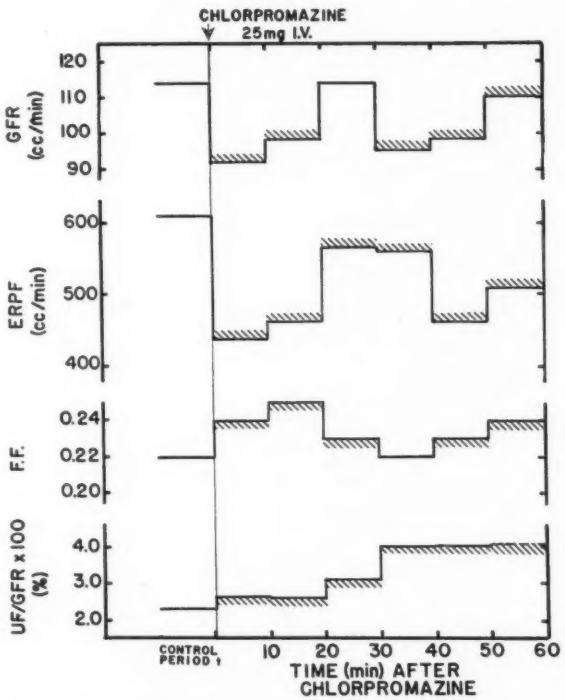

Fig. 1.—Effect of intravenous chlorpromazine on glomerular filtration rate (GFR), effective renal plasma flow (ERPF), filtration fraction (FF), and the ratio of urine flow to filtration rate (UF/GFR ×100).

†Average of three periods.

an average effective renal plasma flow of 616 ± 177 ml. per minute with an average variation of 50.0 ± 31.2 ml. per minute; and an average filtration fraction of 0.22 ± 0.06 with an average variation of 0.016 ± 0.012. Following the intravenous administration of 25 mg. of chlorpromazine there was a prompt drop in the GFR, ERPF, and a rise in the filtration fraction. In each instance there was a return toward the control level in 20 to 30 minutes followed by a

TABLE

PATIENT	AGE	CONTROL						10 MIN.			20 MIN.		
		GFR*	D*	ERPF*	D*	FF	D	GFR	ERPF	FF	GFR	ERPF	FF
S. Z.	50	130	14.5	1,020	90.0	0.13	0.01	-54	-648	0.07	-35	-533	0.05
J. Ca.	53	105	9.5	439	9.3	0.24	0.03	-44	- 64	-0.07	- 3	- 76	0.04
A. B.	30	142	2.0	416	47.0	0.34	0.05	20	- 61	0.11	3	-168	0.24
P. R.	20	96	1.5	618	7.5	0.16	0.01	2	7	0	9	82	-0.01
J. M.	25	100	3.1	632	22.3	0.16	0.01	-23	-187	0.01	-38	-370	0.07
J. F.	35	97	4.5	431	40.3	0.23	0.01	- 2	-104	0.02	- 8	- 51	-0.01
B. S.	35	128	11.1	745	81.7	0.18	0.01	-16	-130	0	-32	-257	0.02
M. J.	35	119	13.6	431	31.3	0.28	0.01	-29	- 55	0	-26	- 22	-0.04
E. K.	44	106	9.6	806	109.7	0.13	0.01	-46	-444	0.04	6	10	0.01
J. H.	35	104	5.1	666	67.3	0.16	0.01	-29	-165	-0.01	- 5	- 30	0
M. P.	53	108	4.0	505	30.3	0.23	0.02	2	- 86	0.03	11	- 68	-0.06
J. C.	21	116	24.0	690	63.7	0.24	0.01	-44	-200	-0.02	-69	-349	0
Mean	—	114	8.5	616	50.0	0.22	0.016	-22	-178	-0.02	-16	-153	0.03
S. D.	—	14	6.3	177	31.2	0.06	0.012	22	178	0.04	24	178	0.08
"p"	—	—	—	—	—	—	—	0.05	0.02	—	—	—	—

*Milliliter per minute (corrected to standard surface area of 1.72m²).
GFR = Glomerular filtration rate (C_{IN}).
ERPF = Effective renal plasma flow (C_{PAH}).
FF = Filtration fraction (C_{IN}/C_{PAH}).
D = Average deviation from the control mean in each individual.

secondary fall of GFR, ERPF, and rise in filtration fraction. The maximum response to the drug varied from patient to patient from 10 to 60 minutes and was statistically significant (Table I).

TABLE

PATIENT	CONTROL						10 MIN.			20 MIN.		
	UF/GFR*	D*	C_{Na}/GFR*	D*	C_K/GFR*	D*	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}
S. Z.	0.79	0.21	0.9	0.19	4.6	0.83	0	0.1	1.0	0.58	-0.1	0.5
J. Ca.	2.94	0.09	3.2	0.30	16.2	0.70	0.18	0	3.8	-1.14	-1.6	-4.2
A. B.	4.02	0.89	2.4	0.02	13.2	0.20	0.44	-0.3	-1.3	1.30	-0.2	-2.7
P. R.	1.07	0.08	1.4	0.05	4.6	0.25	-0.11	-0.1	-0.1	-0.08	0	-0.1
J. M.	2.00	0.65	1.2	0.05	15.6	0.43	1.02	0.1	2.0	-0.20	0	-1.6
J. F.	0.88	0.04	1.3	0.07	11.0	0.83	0.07	0.1	-2.3	0.36	0.5	-0.1
B. S.	1.24	0.45	0.9	0.08	16.6	7.23	2.26	0.1	12.1	3.86	0.2	15.8
M. J.	7.80	0.77	1.6	0.12	21.4	1.60	0.90	0	2.7	-2.10	-0.1	0.9
E. K.	1.28	0.12	1.2	0.08	15.1	0.93	0.44	-0.1	3.1	0.72	0.2	6.0
J. H.	0.93	0.24	0.3	0.02	22.8	1.60	0.45	0	1.4	1.35	0	-3.5
M. P.	3.51	0.79	1.6	0.09	19.1	0.37	0.40	0	-3.7	1.19	-0.1	-2.4
J. C.	1.27	0.14	5.5	0.77	16.0	1.13	0.48	3.9	1.5	0.38	3.4	-0.2
Av.	2.31	0.37	1.8	0.15	14.7	1.34	0.54	0.3	1.6	0.52	0.2	0.2
S. D.	2.00	0.61	1.3	0.20	7.7	1.83	0.61	1.25	3.8	1.40	1.2	5.2
"p"										0.05		

Av. = Average.
* = Per cent.
UF/C_{IN} = Urine flow/glomerular filtration rate ×100.
C_{Na}/C_{IN} = Clearance of sodium/glomerular filtration rate ×100.
C_K/C_{IN} = Clearance of potassium/glomerular filtration rate ×100.
D = Deviation from the control mean in each individual.

The results of the effect of intravenous chlorpromazine in the same 12 patients on urine flow, sodium, and potassium excretion is tabulated in Table II. These are calculated as the ratios of urine flow to inulin clearance (UF/C_{IN}),

I

CHANGE FROM CONTROL MEAN IN EACH INDIVIDUAL															
30 MIN.			40 MIN.			50 MIN.			60 MIN.			MAX.			
GFR	ERPF	FF	GFR	ERPF	FF	GFR	ERPF	FF	GFR	ERPF	FF	GFR	ERPF	FF
16	-235	0.04	-65	-220	-0.06	-22	-186	-0.01	-50	-416	-0.01	-65	-648	0.06
-26	- 84	-0.02	-19	- 11	-0.04	—	—	—	—	—	—	-44	- 84	0.04
-12	- 25	-0.01	- 9	84	-0.07	- 6	124	-0.09	0	304	-0.14	-12	-168	0.24
37	274	-0.01	3	147	0.03	- 2	-108	0.03	- 7	2	-0.02	- 7	-108	0.03
10	-132	0.06	45	-146	0.23	-14	-246	0.06	-14	-374	0.17	-38	-374	0.23
-17	- 56	-0.02	-12	39	-0.01	- 9	- 31	-0.02	10	- 33	-0.02	-17	-104	0.02
- 5	-103	0.01	-12	-143	0.01	-21	-258	0.04	-10	-178	0.03	-32	-258	0.04
-15	- 56	0	0	- 41	-0.03	18	- 33	0.06	20	116	0.07	-29	- 56	0.07
22	32	0.02	19	110	0.01	-10	-183	0.02	49	- 50	0.07	-46	-444	0.07
-20	-195	0.02	- 8	-113	0.01	-44	-326	0.02	- 5	-150	0.03	-44	-326	0.03
-24	-163	0.02	-36	-195	0	-23	-147	0.01	0	- 69	0.02	-36	-195	0.03
33	126	-0.03	-10	-190	0.04	-42	-296	0.03	-37	-313	0.06	-69	-349	0.06
0	- 51	0.01	- 9	- 57	0	-16	-154	0.01	- 4	-106	0.02	-37	-260	0.08
22	136	0.03	26	124	0.07	16	148	0.04	25	204	0.07	19	169	0.07
—	—	—	—	—	—	—	0.02	—	—	—	—	0.01	0.01	0.01

sodium clearance to inulin clearance (C_{Na}/C_{IN}), and potassium clearance to inulin clearance (C_K/C_{IN}), during each collection period, and are expressed as the per cent of the glomerular filtration rate (C_{IN}). During the control periods there was an average UF/C_{IN} of 2.31 ± 2.00 per cent with an average variation from the control means in each individual of 0.37 ± 0.61 per cent; an average C_{Na}/C_{IN} of 1.8 ± 1.3 per cent with an average variation of 0.15 ± 0.20 per cent;

II

CHANGE FROM CONTROL AVERAGE															
30 MIN.			40 MIN.			50 MIN.			60 MIN.			MAX.			
UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}	UF/C_{IN}	C_{Na}/C_{IN}	C_K/C_{IN}
0.89	-0.1	4.0	1.57	0.2	1.7	0.60	0.1	0.6	0.05	0.1	0.6	1.57		
-1.10	-1.1	-2.8	-0.38	-0.7	-1.3							0.18		
0.25	0.6	2.7	-1.26	0.6	3.7	-2.17	0.4	2.2	-1.52	0.7	6.5	1.30		
1.15	0	0.2	3.88	-0.1	1.6	5.93	0	3.9	6.17	0.1	1.8	6.17		
1.48	-0.1	-3.2	3.03	-0.4	-8.4	8.00	0.1	-8.2	9.15	0.3	-5.9	9.15		
0.24	0.5	0	0.08	0.2	-1.0	0.21	0	-3.4	0.71	-0.1	-2.7	0.71		
4.36	0	15.4	6.06	0	12.5	6.61	0.1	12.9	1.24	0	10.3	6.61		
-1.65	-0.5	-5.9	-0.40	-0.5	-5.5	-0.70	-0.6	-8.0	0	-0.4	-5.6	0.90		
1.07	0	3.9	3.97	0.1	5.6	4.12	-0.1	3.6	4.97	-0.3	-2.5	4.97		
1.79	0	-5.7	0.51	0	-9.8	-0.13	0	-9.6	-0.69	0	-16.0	1.79		
0.30	-0.2	-2.8	-1.01	-0.2	-3.4	-0.86	-0.2	-2.5	-1.29	-0.1	-2.9	1.19		
1.25	0.7	-4.7	4.19	3.7	-5.6	4.01	5.1	-4.3	0.55	3.1	-4.4	4.19		
0.83	0	0.1	1.69	0.2	-0.8	1.72	0.4	-1.2	1.76	0.3	-1.9	3.23		
1.43	0.46	1.80	2.73	1.1	6.1	3.57	1.48	6.35	3.29	0.94	6.5	2.79		
0.05			0.01			0.01			0.01			0.01		

and an average C_K/C_{IN} of 14.7 ± 7.7 per cent with an average variation of 1.34 ± 1.83 per cent (Table II). Following chlorpromazine the only statistically significant change was an increase in urine flow with an accompanying rise in the UF/C_{IN} (Fig. 1). The urine flow rose to an average maximum of 5.1 per cent of the glomerular filtration rate (C_{IN}) representing an actual rise in urine flow from an average control level of 2.6 ml. per minute to an average maximum of 7.0 ml. per minute.

The paradoxical finding of a decrease in renal functions associated with an increase in urine output suggests that either there is a direct effect of chlorpromazine on the tubules, an indirect effect on tubular function such as by a decrease in posterior pituitary activity, or both.

In order to investigate this further, we undertook the second part of the study.

Fig. 2 summarizes the results of the assays for antidiuretic substance in the urines of the patients studied. There was a statistically significant difference ($p. < 0.01$) between the control group of patients and the group receiving chlorpromazine, indicating a decrease in excretion of antidiuretic hormonelike substance in the urine following the drug. It is assumed that this substance is at least in part of posterior pituitary origin.

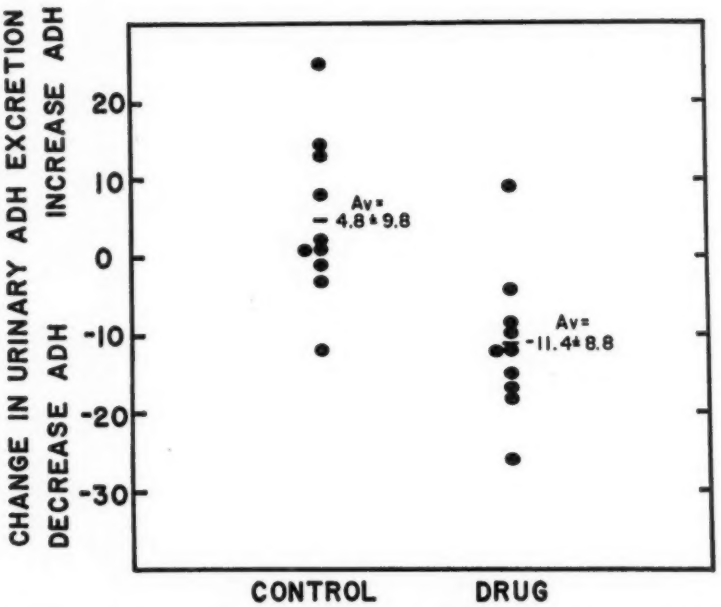

Fig. 2.—Effect of chlorpromazine on the excretion of ADH-like substances in the urine.

DISCUSSION

From the results presented it would appear that chlorpromazine has a marked effect on renal hemodynamics when given intravenously, reducing filtration rate and blood flow and increasing renal resistance, even though no change in blood pressure occurred. This effect would seem to be transient since Moyer[1] has reported that in one hour's time renal function is normal and since our data show that renal function returns toward normal in one hour. Since both the filtration rate and the effective renal plasma flow fall and the filtration fraction rises, apparently there is generalized renal vasoconstriction involving both the efferent and afferent arterioles. There does not seem to be evidence in our data that this is due to anything other than a direct action of the drug on the kidney.

A decrease in an ADH-like substance in the urine, as suggested by the second part of the experiment, probably would not produce the changes seen in renal function. Neither exogenous pitressin[7] nor inhibitions of ADH excretion by the injection of water or alcohol[8, 9] seems to affect renal function.

The ratios C_{Na}/C_{IN} suggest that there is a fall in excretion of Na which is related to the fall in filtration rate.

The ratio C_K/C_{IN} would be expected to rise since K^+ excretion is related more to tubular function than to glomerular filtration. The fact that this did not occur raises the possibility that potassium excretion by the tubule is decreased, either due to the action of chlorpromazine or to the inhibition of the posterior pituitary.

The rise in urine flow/C_{IN} would indicate that a decrease in the tubular absorption of water had occurred. This could be either a direct effect of chlorpromazine on the renal tubule or an indirect effect by way of inhibition of ADH production. The finding of a decrease in concentration of ADH-like substance in the urine in patients receiving chlorpromazine would be compatible with such an inhibition. There is no evidence in our data to indicate which part of the supraopticohypophyseal system is affected.

SUMMARY

Intravenous chlorpromazine produces a rapid decrease in effective renal plasma flow and glomerular filtration, with no change in the C_{Na}/C_{IN} or C_K/C_{IN} ratios. There is a concomitant rise in urine output and a fall in the urinary excretion of an antidiuretic substance. This suggests that the increased urine flow/inulin clearance observed may be due to an inhibition of an antidiuretic-like hormone.

We wish to acknowledge the technical help of Mrs. Ruth A. Perloff in this study.

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THE TREATMENT OF STRYCHNINE POISONING WITH MEPHENESIN

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STRYCHNINE and nux vomica are used in therapeutics as stimulants and tonics. Poisoning with strychnine usually follows the oral intake of large quantities of preparations containing the drug. Poisoning most often occurs in young children when they obtain and eat large quantities of candy-coated tablets containing strychnine. For adults, the fatal dose is usually 60 to 90 mg., but with adequate therapy, recovery may take place following ingestion of 250 mg. or more.[1, 2]

In toxic doses strychnine acts both as a stimulant and as a depressant of the central nervous system. The principal symptoms of strychnine poisoning are referable to increased reflex excitability of the spinal cord. Minimal afferent stimuli produce maximal motor responses. The resulting convulsions may involve all of the voluntary muscles including the diaphragm. Tetanic muscular contractions also develop, resulting in opisthotonos. Following the period of muscular contraction, there is depression and muscular relaxation. Another attack usually follows within a few minutes. After a time the spasms become progressively weaker but the remissions become shorter. Death may occur during one of the early spasms because of asphyxia, or it may be a late consequence of exhaustion and general depression. The patient remains conscious and apprehensive until asphyxial coma supervenes.[1-5]

The treatment of strychnine poisoning has four aspects: prevention of asphyxia; removal of unabsorbed drug; reduction of the strength and frequency of the convulsive seizures; and general support of the patient. The stomach is emptied of any unabsorbed drug by means of a stomach tube; chemical emetics may be depressing and should not be relied upon. A soluble barbiturate is administered intravenously in a dose which is adequate to diminish or suppress the convulsions. The patient is kept in a dark, quiet room in order to minimize extraneous stimuli.[1, 2, 6, 7]

Asphyxia is caused by the sustained contractions of the intercostal muscles, the diaphragm, and the muscles of the air passages. Asphyxia may be prevented by abolishing the convulsions, while maintaining a clear airway and adequate respiratory ventilation. If asphyxia does occur, a clear airway is obtained and artificial respiration with oxygen is given. If convulsions are severe, such large doses of barbiturates may be necessary that they may cause toxic effects and contribute to the general depression, particularly to depression of respiration.

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A newer approach to the treatment of convulsive disorders is the use of muscle-relaxant drugs, such as curare. Of these, mephenesin seems most suitable for the treatment of strychnine poisoning.[8-12]

Mephenesin (Myanesin, Tolserol) is a synthetic chemical which may be administered intravenously to produce muscular relaxation during anesthesia. It is also used for the treatment of spastic diseases and the tremors of alcoholism. Berger[9, 10] found that mephenesin selectively antagonized the effect of strychnine in doses which alone produced no effect upon laboratory animals. The amount of mephenesin necessary to depress reflex hyperexcitability is considerably smaller than that which is needed to depress normal physiologic function. Protection of an animal from the effects of a lethal dose of strychnine could be obtained by the administration of 1/30 of the median lethal dose of mephenesin.

Berger[9, 10] showed that the antagonism between strychnine and mephenesin was more complete than the antagonism between strychnine and Evipal, a short-acting barbiturate. As an antagonist of strychnine, mephenesin has the advantage over barbiturates in that it does not cause central nervous system depression. The principal site of action of strychnine is the spinal cord. Mephenesin also exerts its effect upon the internuncial neurons of the spinal cord and brain stem. In this regard, mephenesin has an advantage over curare and similar drugs, which produce muscular relaxation by interfering with the transfer of impulses at the myoneural junction.

Mephesesin is marketed in 10 per cent solution as Myanesin and in 2 per cent solution as Tolserol, as well as in tablet form. For reducing muscular rigidity, the drug is administered orally in a dose of 3 to 5 Gm. per day. The quantity required to produce consistent relaxation in adults during anesthesia is 1 Gm. to 1.5 Gm. intravenously. The occurrence of serious toxic effects may be avoided by the slow administration of minimal effective amounts of the drug.[13-16]

CASE REPORT

A two-year-old white girl was brought to the emergency room in convulsions, with a history of having ingested an unknown but large quantity of candy-coated strychnine tonic tablets shortly before noon. About twenty minutes later the patient's mother noticed that she began to "act strange." At 12:20 P.M. she gave the child ipecac; shortly thereafter the child vomited and then fell over backward, striking her head upon the floor. She was apparently unhurt and jumped up immediately, but she then began to have abnormal jerky movements.

She was brought to the hospital by ambulance and arrived in the emergency room at 1 P.M. At this time she was observed to be unconscious, cyanotic, in profound opisthotonos, and there was spasmodic jerking of the extremities. She was immediately given 60 mg. of sodium phenobarbital intramuscularly by the intern in the emergency room, without apparent effect. The Anesthesia Department was called and at 1:15 an infusion was started and the patient was given 2.5 ml. of Myanesin (250 mg. mephenesin) slowly intravenously. Before the injection was completed, the jerking movements ceased, and the muscular rigidity and opisthotonos disappeared. Breathing and color became normal and consciousness returned.

Gastric lavage was done for the purpose of removing any additional quantities of the drug which remained in the stomach. Blood and urine specimens, as well as several of the tablets, were sent to the toxicology laboratory for analysis. The child was restless and she cried, but she had no further convulsive movements or twitching during the stimulus of the gastric lavage, venipuncture, and catheterization.

At 2 P.M. she was given 60 mg. of sodium phenobarbital slowly intravenously. She then fell asleep and awakened at intervals, screaming and restless. At 2:25 P.M. she was given an additional 2 ml. Myanesin (200 mg. mephenesin) intravenously. Following this she again became quiet. An infusion of 5 per cent dextrose in normal saline was allowed to run slowly throughout the day. In the evening 60 mg. of sodium phenobarbital was again given for sedation. On the following day a thorough examination showed her to be normal in every respect, and she was discharged from the hospital.

Biologic and chemical analyses were performed by the toxicology laboratory. The tablets each contained 1.5 mg. of strychnine. The blood and urine which were submitted for examination showed the presence of strychnine on both chemical and biologic analysis.

COMMENT

The treatment of strychnine poisoning with mephenesin was based upon laboratory evidence that this drug antagonizes the effects of strychnine in animals. Strychnine produces its symptoms by increasing the reflex excitability of the spinal cord. Myanesin produces its effect by decreasing the reflex excitability of the spinal cord. The use of the one drug to antagonize the other seems logical; the decision to do this had been made long before this patient was admitted.

In the case cited, the history and physical findings were typical of strychnine poisoning. The effectiveness and speed of action of mephenesin were exceedingly satisfactory. Following the injection, the convulsions and the opisthotonos disappeared, while at the same time there was no decrease in the respiratory activity of the patient. On the contrary, respiration and oxygenation improved, as the spasm of the respiratory muscles disappeared. Twitching and convulsions did not reappear even during the stimulation of gastric lavage, catheterization, and venipuncture. The barbiturates were used to produce mild sedation during the period of hyperexcitability but were of no immediate value in the treatment of the acute convulsive episode.

The technique used for the administration of mephenesin is that of very slow intravenous injection, so that the effect of drug is observable and an overdose is avoided.

SUMMARY

Strychnine poisoning and its treatment are discussed. A case of strychnine poisoning in a two-year-old girl is presented. Treatment consisted of the intravenous administration of mephenesin for the control of convulsions and opisthotonos, and sodium phenobarbital for the control of restlessness. Further investigation of the use of mephenesin as an anticonvulsant, particularly following strychnine poisoning, is recommended.

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LABORATORY METHODS

A NEW METHOD FOR THE DETERMINATION OF SERUM IRON-BINDING CAPACITY. I

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WITH THE TECHNICAL ASSISTANCE OF ANNE P. TRAKAS

A SIMPLE chemical procedure, using an ion exchange resin, is described for the determination of total serum iron-binding capacity (TIBC). It is applicable to fresh or stored sera, and to lipemic or icteric specimens.

It is difficult or impossible to apply optical procedures[1, 2] to sera which are not optically clear. Seeking a more generally applicable method, we tested the chemical procedure of Laurell[3] but were not able to achieve satisfactory precision and accuracy. A recently published method[4] employing ammonium sulfate precipitation after addition of radioactive iron was also investigated. Results were consistently 35 to 115 mcg. per 100 ml. higher than values obtained on the same sera by the optical method, and by the addition of radioiron to previously saturated sera, it was possible to show that this error was caused by adsorption of 15 to 25 per cent of the excess unbound iron by the precipitate and filter paper.

Attempts were made to obtain saturated serum by adding iron as the ferrous ion and removing the unbound excess by cation exchange resins. Tests with a number of resins under a variety of conditions were unsuccessful—either the resin failed to remove all of the excess iron, or it removed some of the iron bound by the serum protein. However, an anion exchange resin, Amberlite IRA 410, was found to remove excess iron when the iron was added as ferric ammonium citrate, and not to remove any of the protein-bound iron. The action of the anion exchange resin apparently is to bind one of the carboxyl groups of citrate in exchange for chloride, and the iron remains attached to the other carboxyls.

In the method described in this article, 1 ml. of serum is saturated by adding an excess of ferric ammonium citrate. The iron which is not bound by the serum proteins is then removed by addition of a small amount of IRA 410 buffered

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at pH 7.5 on the chloride cycle. After dilution and centrifugation, an aliquot of the supernatant is analyzed for iron content, giving a measure of the total iron-binding capacity of the serum.

A preliminary report of the early part of this work was made,[5] at which time treatment with resin was effected by passing the saturated serum through a small column. Addition of the resin directly to the serum in a centrifuge tube has since been found to give equally valid results, and to be a simpler technique for clinical use.

Data are presented on the ability of the resin treatment to remove all of the unbound iron while retaining none of the iron bound to the serum protein. Results are compared with those obtained by the optical method, and values are given for the iron-binding capacity of a series of normal and pathologic sera.

METHODS

Procedure for the Determination of Serum Iron-Binding Capacity.—

Reagents: 1. Ferric ammonium citrate, 0.050 mg. Fe per milliliter. Commercial preparations appear to contain some iron as the hydroxide, so this reagent is prepared locally. A known amount of $FeCl_3$ is precipitated with ammonium hydroxide, centrifuged, dissolved with a few crystals of citric acid with heat, adjusted to pH 6.5 to 7 with dilute ammonium hydroxide (bromthymolblue indicator), and diluted to volume. The solution is kept under refrigeration and appears to be stable, as indicated by a light-green color, for at least one or two months.

2. Buffer, pH 7.5, containing 0.11 M NaCl and 0.04 M barbital (diethylbarbituric acid). "Tris" buffer can probably also be used. Due to the low solubility of barbital at this pH, it is best to approach the pH from the alkaline side. Six and four-tenths grams of NaCl, about 6 Gm. of diethylbarbituric acid, and 2.3 Gm. of its sodium salt are used per liter.

3. Amberlite IRA 410 resin.* The resin is suspended in approximately 3 N HCl overnight to wash it free of iron and to saturate it with chloride; then it is washed well with distilled water. It is brought to pH 7.5 (phenol-red indicator) by suspension in the above buffer, with the addition of a little NaOH if necessary. It is then dried at 95° C.

Apparatus (made iron-free by rinsing in 6 N HNO_3):

1. Centrifuge tubes, 15 ml. capacity.

2. Test tubes, approximately 1.5 × 12.5 cm.

3. Pipettes, 0.1, 1, and 5 ml. capacity.

4. Stirring rods (wooden applicator sticks have been found to be satisfactory without acid rinsing.)

Procedure: 1. Pipette a 1.0 ml. sample of serum or heparinized (not citrated or oxalated) plasma into a centrifuge tube. Add 0.1 ml. of the ferric ammonium citrate solution (equals 5 mcg. of iron). Let stand about 10 minutes at room temperature.

2. Add 0.4 ml. of the dry resin and stir occasionally for 5 to 10 minutes. Then add 5.0 ml. of buffer and continue to stir for several minutes. Centrifuge briefly and remove 5.0 ml. of the supernatant, using a manual pipette control to avoid disturbing the resin.

3. Place the 5 ml. of the supernatant in a 1.5 × 12.5 cm. tube and analyze for iron content.

*Available from Rohm and Haas, Philadelphia.

The iron analysis can be performed by any of several methods, provided that it can be accomplished without an appreciable increase in volume, since the serum has already been diluted. Consideration of the buffer present may be required. The method for serum iron content given in the following paper[6] has been found to be well suited for this purpose.

Calculation: The value obtained for the iron content of the 5 ml. aliquot, multiplied by 6/5 × 100, gives the total iron-binding capacity per 100 ml. of serum (TIBC). Subtraction of the serum iron content, determined separately, gives the unsaturated iron-binding capacity (UIBC) if desired. For precise work a small correction may be applied for the amount of unbound iron not retained by the resin, shown later in this article to be about 4 per cent of the unbound iron. To apply this correction, subtract 0.04 × (500 − UIBC) from the TIBC obtained.

Blank: A blank determination should be made occasionally, with use of 1 ml. of water in place of serum. In our experience the blank due to resin treatment has never amounted to more than 13 mcg. per 100 ml.

RESULTS

In Tables I and II are presented the results of tests with radioactive iron to show the effectiveness of the resin in removing excess iron while not removing iron bound by serum. The resin retained all but about 4 per cent of the excess iron, while the recovery of protein-bound iron averaged 101 per cent.

TABLE I. EFFECTIVENESS OF RESIN IN REMOVING EXCESS IRON

NUMBER OF DETERMINATIONS	PER CENT OF EXCESS IRON RETAINED BY RESIN		
	MEAN	S. E.	S. D.
20	96.2%	±0.2%	0.9%

A series of sera was treated by the method described, except that just prior to addition of the resin, 0.007 ml. of a solution of Fe^{59}-labeled ferric ammonium citrate was added. The amount of iron did not exceed 0.05 mcg. The percentage of Fe^{59} retained by the resin was determined by gamma ray counting of a 5 ml. aliquot of the supernatant in comparison with suitable standards.

In these and other experiments employing Fe^{59}-ferric ammonium citrate, it was found that exchange between the iron bound by serum and the excess iron occurs at a slow rate. Hence saturated samples were not allowed to stand longer than a few minutes prior to resin treatment. Furthermore, the rather large amount of iron occurring in the IRA 410 resin, even after acid washing, is apparently made available for exchange with iron bound to serum by the acid pretreatment. Therefore resin was used without acid washing for Fe^{59} studies, while the acid-washed resin was used for TIBC determinations.

TABLE II. RECOVERY OF IRON BOUND TO SERUM PROTEIN

NUMBER OF DETERMINATIONS	PER CENT OF PROTEIN-BOUND IRON RECOVERED		
	MEAN	S. E.	S. D.
12	101.3%	±0.8%	2.8%

The iron-binding protein of a series of sera was labeled by addition of 0.007 ml. (0.05 mcg. Fe) of Fe^{59}-labeled ferric ammonium citrate. The sera were incubated at 38° C. for 15 to 30 minutes to insure binding and were then saturated by adding 0.1 ml. (5 mcg. of Fe) of nonradioactive ferric ammonium citrate solution. Resin treatment was carried out by the technique described. The recovery of Fe^{59} was determined by counting a 5 ml. aliquot of the supernatant in comparison with suitable standards.

The precision of the method is illustrated by the results of 11 analyses made on the same serum specimen (Table III). Range of values was 282 to 315 mcg. per 100 ml., with a standard deviation of 10 mcg. per 100 ml.

TABLE III. REPRODUCIBILITY OF RESULTS

NUMBER OF DETERMINATIONS	SERUM IRON-BINDING CAPACITY (MCG./100 ML.)		
	MEAN	S. E.	S. D.
11	295	±3	10

A single serum specimen was analyzed repeatedly by the procedure described in the text.

The effect of increasing the amount of resin employed is shown in Table IV. No significant improvement in effectiveness is seen until 1 ml. of resin is used, and the amount of improvement obtained did not compensate for the larger volumes of buffer required to rinse the serum from the resin.

TABLE IV. EFFECTIVENESS OF INCREASED AMOUNTS OF RESIN

ML. RESIN EMPLOYED	PER CENT OF EXCESS IRON RETAINED BY RESIN
0.4	95.7
0.6	96.1
0.8	96.0
1.0	97.1

A single specimen of pooled sera was tested by the procedure given under Table I. Results are the average of 2 or more tests.

A series of normal and pathologic sera was analyzed by the optical method of Rath and Finch[1] and by the resin method. Serum iron content was determined by the procedure described in the following paper.[6] Results are given in Table V.

TABLE V. ANALYSES OF SAME SERA BY RESIN AND OPTICAL METHODS

TYPE OF SERUM	SI (MCG./100 ML.)	TOTAL IRON-BINDING CAPACITY (MCG./100 ML.)		
		OPTICAL METHOD[1] (UIBC + SI)	RESIN METHOD	RESIN-Fe^{59} METHOD* (UIBC + SI)
Normal	119	262	274	275
Normal	123	363	345	354
Normal	194	262	286	310
Normal	142	292	320	321
Normal	97	353	381	382
Normal	72	414	420	428
Normal	194	278	355	333
Normal	120	362	393	
Normal	102	292	348	
Fe-deficient	26	442	449	
Fe-deficient	23	360	358	
Malignancy	40	152	172	
Treated hemochromatosis	43	225	279	290
Hemochromatosis	235	265	262	282

*See text.

The agreement between the resin and optical methods was about ± 30 mcg. per 100 ml. Results with the resin method tended to be slightly higher, but the average difference of 23 mcg. per 100 ml. is of doubtful significance.

In addition to the determination of the TIBC by resin treatment, in most instances labeled ferric ammonium citrate solution was used which had been prepared from $FeCl_3$ containing $Fe^{59}Cl_3$. By multiplying the per cent of the Fe^{59} which was not retained by the resin by the amount of iron added (5 mcg.), one is able to obtain an independent figure for the unsaturated iron-binding capacity (UIBC). These figures are reported in Table V together with the results of the standard resin and optical methods. The TIBC values obtained by the chemical method agreed well with the values obtained by adding the serum iron content to the UIBC determined with Fe^{59}. This is a further check on the validity of the resin procedure.

In Table VI are listed statistical data on 34 normal sera and individual values of 10 pathologic specimens determined by the resin method. The average normal value, 333 mcg. per 100 ml., is in good agreement with the results obtained by others with other methods.[1, 3, 7-9] Two-thirds of normal sera showed TIBC's in the range of 277 to 379 mcg. per 100 ml., but the extreme values ranged from 248 to 422 mcg. per 100 ml.

TABLE VI. IRON-BINDING CAPACITY OF NORMAL AND PATHOLOGIC SERA

NORMAL SERA					
	NUMBER OF PERSONS	MEAN	S. E.	S. D.	RANGE
TIBC	34	333	±8	46	248-422
SI	34	119	±7	41	57-194

PATHOLOGIC SERA		
TYPE OF SPECIMEN	SI	TIBC
Fe-deficient	35	424
Fe-deficient	27	385
Fe-deficient	26	449
Fe-deficient	23	358
Malignancy	40	172
Nephrotic syndrome	70	220
Nephrotic syndrome	70	258
Nephrotic syndrome	89	278
Liver disease	202	304
Hemochromatosis	235	262

All values are mcg./100 ml.

Results on pathologic specimens were in agreement with findings of others.[1, 3, 7-9] TIBC was found to be elevated in iron-deficiency anemia and reduced in hemochromatosis, nephrotic syndrome, and malignancy.

SUMMARY

1. A simple chemical procedure for the determination of serum iron-binding capacity is described. It requires a sample of only 1 ml. and is applicable to fresh or stored sera and to lipemic or icteric specimens.

2. The serum is saturated by adding an excess of iron as ferric ammonium citrate. The iron which is in excess of the serum-binding capacity is then removed by addition of a small amount of an anion exchange resin to the centrifuge tube. The serum is diluted and an aliquot of the supernatant analyzed for iron content, which is a measure of the total iron-binding capacity of the sample.

3. Data are presented demonstrating the efficiency of the resin treatment, as shown by studies with Fe^{59}. Values are compared with those obtained on the same specimens by another method, and iron-binding capacity measurements on a series of normal and pathologic sera are given.

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A SIMPLE IMPROVED METHOD FOR THE DETERMINATION OF SERUM IRON. II

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WHILE many methods for the determination of serum iron have been devised,[1-11] we could find none which was at the same time simple, sensitive, consistently productive of complete iron extraction, and applicable to cloudy, jaundiced, or previously frozen serum specimens. Frequently there was disagreement between the results obtained on a single specimen by different methods.

The procedure of Barkan and Walker,[3] in which iron is extracted by addition of dilute HCl followed by trichloracetic acid, is simple in application, but was found to give incomplete recovery of iron from specimens which had been stored in the frozen state. Addition of a reducing agent (thioglycolic acid) together with the HCl gave complete iron recovery in every instance in which it was tested.

This paper describes a simple procedure for serum iron determination utilizing the above principle. Sensitivity is improved over that of most previous methods by use of the recently introduced iron reagent bathophenanthroline. The tris-iron complex of bathophenanthroline has a molar extinction coefficient more than double that of the iron-orthophenanthroline or iron-α-α'-dipyridyl complexes[7, 12] permitting determination of the iron in a 2 ml. specimen of serum. Due to the extreme insolubility of bathophenanthroline in aqueous solutions, color development is carried out in a solution containing 30 per cent alcohol, which eliminates the need for extraction of the colored complex with organic solvents.

The method to be described is particularly well suited for use in conjunction with the determination of serum iron-binding capacity by the procedure described in the preceding paper,[13] since iron extraction can be carried out with only a slight increase in volume of solution.

METHODS

A. *Method for Determination of Serum Iron.*—

Reagents: 1. Thirty per cent trichloracetic acid, distilled to render iron-free.

2. Thioglycolic acid (2-mercaptoethanoic acid) reagent grade, 80 per cent solution in water, in glass-stoppered dropping bottle.

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3. Concentrated HCl, reagent grade, in glass-stoppered dropping bottle.

4. Two-tenths normal HCl, made from reagent grade HCl.

5. Saturated solution of sodium acetate, reagent grade.

6. Bathophenanthroline (4,7-diphenyl-1,10-phenanthroline),* made to 0.02 per cent solution in 3/1 isopropyl alcohol/isoamyl alcohol, reagent grade.

7. Paraffin film.

Glassware (made iron-free by rinsing in 6 N HNO_3):

1. Test tubes, approximately 1.5 × 12.5 cm.

2. Cuvettes for colorimeter or spectrophotometer, minimum readable volume 6.5 ml. or less.

3. Pipettes – 0.5, 1, 2, 3, and 4 ml. capacity. Two milliliter and 4 ml. sizes needed in largest numbers.)

4. Stirring rods. Wooden applicator sticks rinsed in dilute HCl-thioglycolic acid solution have been found satisfactory.

Procedure: 1. Pipette a 2.0 ml. sample of serum or plasma (fresh or stored, citrated, oxalated, or heparinized) into a test tube. Add 3.0 ml. of 0.2 N HCl and 1 drop of thioglycolic acid. Mix by swirling and let stand 30 minutes at room temperature.

2. Add 1.0 ml. 30 per cent trichloracetic acid. Mix with a stirring rod and let stand 15 to 30 minutes at room temperature, covered with paraffin film. Centrifuge for about 15 minutes at high speed.

3. Pipette a 4.0 ml. aliquot of the supernatant into a cuvette. Use of a manual pipette control is recommended to avoid disturbing the precipitate.

4. Add 0.5 ml. of saturated sodium acetate solution and 2.0 ml. of the alcoholic bathophenanthroline solution. Mix by swirling.

5. Read the optical density at 535 mμ or with a green filter, after color development has taken place for 10 to 15 minutes at room temperature, against a blank tube which has been prepared with use of 2.0 ml. of distilled water in place of serum in the above procedure.

Standardization: Use known amounts of Fe in 2.0 ml. of water to replace the serum in the above procedure. (A standard Fe solution can be prepared by dissolving a weighed amount of pure iron wire in HCl and diluting to about 1 mg. Fe per milliliter.) With use of the Coleman Junior spectrophotometer with 19 mm. cuvettes, a straight line relationship between iron concentration and optical density is obtained, with a slope of 0.518 optical density units per microgram of iron per milliliter of the final solution (Fig. 1).

Calculation of results: The serum iron content is calculated by the expression

$$\frac{\text{Optical density}}{\text{Standard O.D. per mcg. Fe/ml.}} \times \frac{6.5 \times 1.5 \times 100}{2.0 \times 1.015} = \text{mcg./100 ml. serum iron}$$

The significance of the various terms is:

6.5—total volume of the final solution;

1.5—correction for 4/6 aliquot of supernatant taken;

100—percentage factor;

2.0—correction for 2 ml. sample of serum;

1.015—correction for the volume occupied by the solids upon precipitation of the proteins. This value was determined experimentally by measuring the recovery of added Fe^{59}, as described later in this article.

With use of the standard density value of 0.518, determined on the Coleman spectrophotometer, this calculation reduces to

927 × optical density = mcg./100 ml. serum iron.

*Available from G. Frederick Smith Chemical Company, Columbus, Ohio.

Modified procedure for use with resin-saturation method for serum iron-binding capacity determination (previous paper[13]):

To the 5.0 ml. of serum plus buffer obtained after resin treatment, add one drop of concentrated HCl in place of the 3.0 ml. of 0.2 N HCl. Then proceed with the addition of thioglycolic acid and the balance of the procedure as described. Since only 1.0 ml. of serum is used and a 5/6 aliquot taken, the calculation becomes

$$\frac{\text{Optical density}}{\text{Standard O.D. per mcg. Fe/ml.}} \times \frac{1.5 \times 6 \times 6.5 \times 100}{5 \times 1.015} = \text{mcg./100 ml. total iron-binding capacity (TIBC)}$$

with use of the standard density value of 0.518, this reduces to 2,210 × optical density = mcg./100 ml. TIBC.

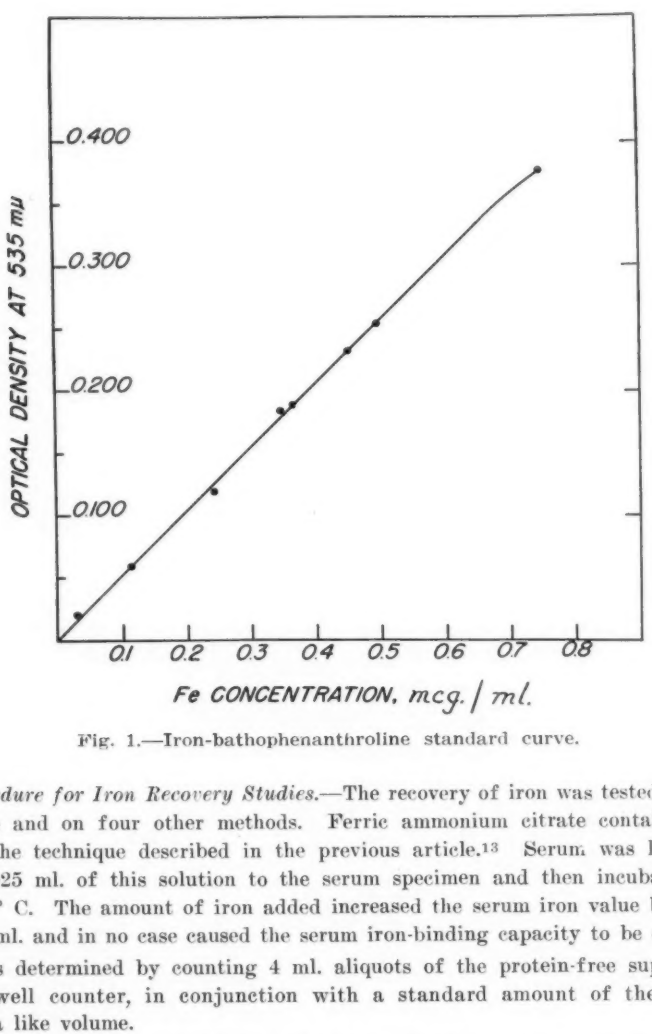

Fig. 1.—Iron-bathophenanthroline standard curve.

B. *Procedure for Iron Recovery Studies.*—The recovery of iron was tested on the method described here and on four other methods. Ferric ammonium citrate containing Fe^{59} was prepared by the technique described in the previous article.[13] Serum was labeled in vitro by adding 0.025 ml. of this solution to the serum specimen and then incubating it for 30 minutes at 38° C. The amount of iron added increased the serum iron value by only 5 to 20 mcg. per 100 ml. and in no case caused the serum iron-binding capacity to be exceeded.

Fe^{59} was determined by counting 4 ml. aliquots of the protein-free supernatants in a scintillation well counter, in conjunction with a standard amount of the Fe^{59} solution contained in a like volume.

The iron recovery was determined colorimetrically in 5 instances, after adding an amount of nonlabeled ferric ammonium citrate sufficient to increase the serum iron value 133 mcg. per 100 ml. and incubating as above.

In order to check whether the technique of adding labeled iron to the serum affected the recoveries with the present method, serum was also labeled physiologically by feeding Fe^{59}. A normal fasting male subject was fed 4 mg. of iron as $FeCl_3$, containing 20 μc of Fe^{59}. The iron was reduced prior to feeding by addition of 200 mg. of ascorbic acid. Serum samples were obtained at 2½ and 4½ hours following feeding. Fe^{59} extraction was determined by comparing the counts in a 2 ml. sample of untreated serum with those in the protein-free supernatant.

RESULTS

Conditions of Color Development With Bathophenanthroline.—The standard curve (Fig. 1) is linear to about 0.6 mcg. Fe per milliliter, or 0.0107 millimoles per liter, at which point the molar ratio of bathophenanthroline to iron is 17/1. This iron concentration represents a serum iron content of slightly over 300 mcg. per 100 ml. Should a serum be encountered with a higher iron content than 300 mcg. per 100 ml., the analysis should be repeated with use of 1 ml. of serum and 1 ml. of distilled water in place of 2 ml. of serum.

The blank tube, read against a tube containing distilled water, should not exceed an equivalent serum iron of 10 to 15 mcg. per 100 ml. (optical density of about 0.012 with use of the Coleman). The major contribution in our experience was from the thioglycolic acid; the blank value could be reduced by half, if desired, by distilling this reagent.

The color of the iron-bathophenanthroline complex appears almost instantly on mixing, and color development is complete within 5 minutes. The absorbance was observed to be constant for at least 24 hours.

The pH resulting after addition of the saturated sodium acetate solution is about 4.3. The absorbance of the iron-bathophenanthroline complex has been found to be constant over a pH range of 1.3 to 9, although below pH 2, color development is slow, and at pH 9, the color faded on standing.

Specificity of the Iron Extraction.—In Fig. 2 the absorption spectra of solutions resulting from serum iron determinations are compared with the spectra of the pure iron-bathophenanthroline and copper-bathophenanthroline complexes under similar conditions. The iron-bathophenanthroline curve shows a fairly sharp peak at 535 mμ. The millimolar absorbance of 22.4 at this wave length agrees with the value reported by others.[7, 12, 14] It is seen that copper is not removed from serum in significant amounts by the HCl and thioglycolic acid treatment and that iron is apparently the only substance present giving color with bathophenanthroline. Cobalt gave no color with bathophenanthroline under the conditions used here.

The use of reducing agents as strong as thioglycolic acid has been avoided by some workers because under some conditions it extracted iron from hemoglobin.[5] Tests on the proposed method showed that the presence of 30 mg. per 100 ml. of hemoglobin caused an increase in the serum iron value of only 5 mcg. per 100 ml., corresponding to extraction of about 5 per cent of the iron from hemoglobin. This amount of hemoglobin in serum is detectable to the unaided eye, hence specimens not visibly pink can be analyzed safely by this method.

Although ascorbic acid used in place of thioglycolic acid was found to remove essentially no iron from hemoglobin, it did cause extraction of copper from serum, which gave added color with bathophenanthroline under the conditions employed. The color due to copper could be avoided by keeping the pH of the final solution below 2, but the development of the color with iron was slower as the pH was reduced, and buffering of the final solution became extremely critical.

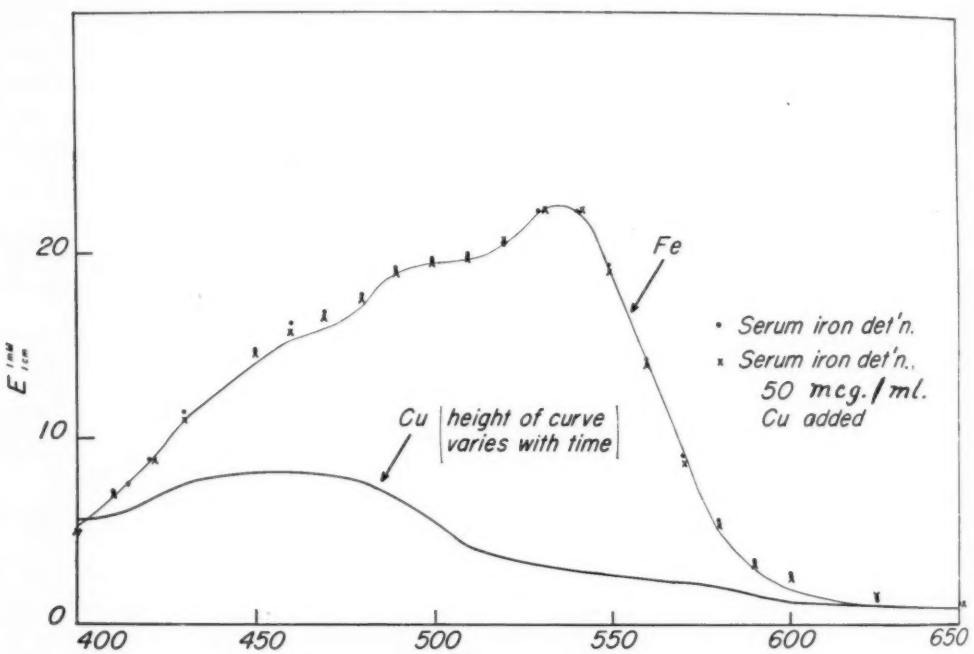

Fig. 2.—Absorption spectra of solutions obtained by the procedure for serum iron determination described in the text. The smooth curves labeled *Fe* and *Cu* were obtained by adding iron or copper to blank analyses. The points represent actual analyses of serum specimens. The solutions contain 30 per cent 3/1 isopropyl/isoamyl alcohol, 3.1 per cent trichloracetic acid, 0.61 N HCl, 0.062 mg. per milliliter bathophenanthroline, and 2.5 mg. per milliliter thioglycolic acid, neutralized to pH 4.3 with sodium acetate. Optical densities were read in a 1 cm. cuvette in a Beckman spectrophotometer, against a similar blank solution.

The absolute absorbance in the presence of copper was variable with time under these conditions, probably due to transformation between the mono and bis copper complexes.[11] The curve plotted is that obtained at about 75 minutes. The points for serum iron determinations were equated to the curve for iron alone at 535 mμ.

Completeness of Iron Recovery.—Table I summarizes the recoveries observed with this procedure and with the four other methods tested. It is seen that iron is extracted completely by the new technique, regardless of whether the serum is fresh or stored, whether the tracer iron is added in vitro or by oral administration, or whether recovery of added iron is determined chemically or by use of radioisotopes. Of the four alternate methods tested, only the 6 N HCl—trichloracetic acid method of Vahlquist[4] showed complete iron extraction on both fresh and frozen specimens.

TABLE I. SUMMARY OF IRON RECOVERIES BY 5 SERUM IRON METHODS

METHOD TESTED	RECOVERY DET'D BY	CONDITIONS	NO. DET'NS.	MEAN RECOVERY (%)	S. E. (%)	S. D. (%)
New procedure	Added Fe^{59}	All determinations	33	101.5	±0.48	2.8
New procedure	Added Fe^{59}	Fresh specimens	6	103.3	±0.73	1.8
New procedure	Added Fe^{59}	Stored specimens	27	101.1	±0.54	2.8
New procedure	Added Fe^{59}	Oral Fe^{59}	4	103.5	±1.12	2.3
New procedure	Added Fe	All determinations	5	98.2	±0.60	1.3
Barkan and Walker[3]	Added Fe^{59}	Fresh specimens	11	100.5	±0.73	2.4
	Added Fe^{59}	Stored specimens	30	81.2	±1.58	8.5
Kitzes, Elvehjem, and Schuette[5]	Added Fe^{59}	Fresh specimens	27	88.7	±1.59	8.3
	Added Fe^{59}	Stored specimens	4	88.3	±0.20	0.4
Ramsay[8b]	Added Fe^{59}	Fresh specimens	4	94.0	±1.18	2.0
Ramsay[8b]	Added Fe^{59}	Stored specimens	8	94.9	±0.48	1.4
Vahlquist[4]	Added Fe^{59}	Fresh specimens	6	103.6	±0.66	1.6
Vahlquist[4]	Added Fe^{59}	Stored specimens	8	99.2	±0.73	2.1

The over-all recovery of Fe^{59} was 101.5 per cent ± 0.48 per cent. That this value is greater than 100 per cent is believed to be due to the precipitation of the protein as a separate phase. Apparently the 6 ml. of solution resulting after trichloracetic acid addition contains about 1.5 per cent of solids, so that the 4.0 ml. aliquot actually is slightly greater than ⅔ of the total liquid. This consideration has previously been applied to analysis of serum for calcium by Severinghaus and Ferrebee,[15] who subtracted 1 per cent from their observed values as a correction factor.

Normal and Pathologic Serum Iron Values.—In Table II are listed the statistical data on a series of normal, iron-deficient, and hemochromatotic male patients. The normal value of 119.7 mcg. per 100 ml. is in the midrange of the values reported by others.[4, 16-18] This is additional evidence that the results with this method are not influenced by undetected hemolysis. The precision of duplicate analyses was high. Individual results in a series of 24 duplicates averaged only ± 0.8 mcg. per 100 ml. deviation from the average of the two.

TABLE II. SUMMARY OF SERUM IRON VALUES WITH NEW PROCEDURE

TYPE*	NO. OF PERSONS	SERUM IRON CONTENT MCG./100 ML.			
		RANGE	MEAN	S. E.	S. D.
Normal male	43	56-183	119.7	±5.4	35.6
Iron deficient	5	10-26	19.6	±3.2	7.2
Hemochromatotic	3	235-246	241	±3.2	6.0

*Samples were drawn in the morning, usually with the patient in the fasting state.

Values obtained with the proposed method were compared with those obtained with two earlier procedures (Table III). The methods of Kitzes, Elvehjem, and Schuette,[5] and Barkan and Walker[3] gave results on a series of normals 88.5 per cent and 76.5 per cent, respectively, of the values obtained with the new method. Apparently the differences are due to losses in the extraction process, since these percentages approximate the recoveries observed with Fe^{59} (Table I). In three determinations on patients with hemochromatosis,

the method of Kitzes, Elvehjem, and Schuette gave even lower values compared to the method reported here. It has also been observed with values in the high normal range that the discrepancy between the two methods was more pronounced than with lower values.

TABLE III. COMPARISON OF SERUM IRON VALUES WITH THOSE OBTAINED BY OTHER PROCEDURES ON SAME SERA

ALTERNATE PROCEDURE	TYPE OF SPECIMEN	NO. DET'NS	SERUM IRON CONTENT MCG./100 ML.		
			NEW METHOD	ALTERNATE METHOD	% ALT./NEW
Kitzes, Elvehjem, and Schuette[5]	Normal (fresh and stored)	16	104.5	92.5*	88.5
Kitzes, Elvehjem, and Schuette[5]	Hemochromatotic (stored)	3	241	186	77.2
Barkan and Walker[3]	Normal (stored)	4	81	2	76.5

*Some of these determinations were performed by Miss Barbara Williams in the Clinical Laboratory of the Children's Medical Center, Boston.

DISCUSSION

Most of the published methods for serum iron determination utilize first some technique for releasing the iron from its combination with the iron-binding globulin, followed by precipitation of the serum proteins. Strong (2 to 6 N) HCl has been used, particularly by German and Scandinavian workers.[2, 4, 17, 19] This technique has been shown in this study and by Bothwell and Mallett[11] to give complete extraction and to give results agreeing with other methods.[11] However, the large amount of alkali required to neutralize the strong acid causes an appreciable increase in both the final volume and in the blank reading, factors both of which decrease the sensitivity.

Use of dilute (0.2 to 0.3 N) HCl, introduced originally by Barkan,[20] is a satisfactory and simple procedure, but it gives low values when applied to stored serum specimens (Tables I and III). The extraction procedure of the method proposed in this paper is the same as that of Barkan and Walker,[3] except for the introduction of a reducing agent and use of room temperature instead of 38° C. It may be conjectured that fresh serum releases its iron at pH 1 to 2 more completely than does stored serum, because of an oxidative process occurring incident to storing. Incubation at 38° C. was not found to be necessary when a reducing agent was added.

Cartwright and associates[16] reported recoveries of 95 to 103 per cent of added iron with the Barkan and Walker method, but Peterson[7] obtained values of only 83 to 93 per cent of Fe^{59}. He did not state whether the sera were fresh or stored.

Reducing agents have been used previously to release the iron from its combination. Ramsay uses sodium sulfite, and his method apparently gives reasonable values.[8, 11] It showed iron recoveries of only 95 per cent (Table I) or 83 to 110 per cent,[8a] however, and involves the inconveniences of preparing solutions daily and of boiling to precipitate the serum proteins. Peterson[7] adds

thioglycolic acid along with tricholacetic acid, a procedure introduced by Tompsett in 1934.[21] He makes use of the greater color intensity of bathophenanthroline, but the procedure requires heating and extraction of the colored complex with alcohol. He reported 85 to 98 per cent recoveries of added iron.

The method of Kitzes, Elvehjem, and Schuette[5] employs boiling to denature the serum proteins and to release the iron prior to addition of trichloracetic acid. It gave values lower than the proposed method (Table III) and variable iron recoveries averaging under 90 per cent (Table I). Other workers reported 64 to 92 per cent recovery of added iron or radioiron,[7] 95 to 103 per cent recovery of added iron,[16] and values 30 to 60 mcg. per 100 ml. lower than those obtained with Ramsay's method.[8a]

Schade and co-workers[9] have published a procedure in which iron is released by means of ascorbic acid at pH 6, and then the color is developed with terpyridine without protein precipitation and read against a serum blank. While we have had no experience with this method, it appears very simple in application, but would seem to suffer in sensitivity when the serum specimens are turbid or otherwise optically dense.

Determination of iron by digestion or extraction with acid and color development with thiocyanate has been applied,[1,6, 10] but the iron-thiocyanate color is of low intensity and requires special measures such as alcoholic extraction of the complex.

It is felt that the method introduced in this paper improves upon the existing procedures by producing valid results with stored as well as fresh specimens, by greater simplicity of technique, and by greater sensitivity and precision.

SUMMARY

1. An improved method, based on the Barkan and Walker procedure, is described for the determination of serum iron. Two milliliters of serum or plasma are treated with 3 ml. of 0.2 N HCl and a drop of thioglycolic acid at room temperature, and then the proteins are precipitated by trichloracetic acid. The iron in an aliquot of the protein-free supernatant is determined by addition of sodium acetate and an alcoholic solution of bathophenanthroline, which is a reagent twice as sensitive for iron as compounds in general use.

2. This method has been shown to give consistently complete extraction of iron from fresh or frozen serum specimens, in contrast to some earlier methods. Precision is about ± 1 mcg. per 100 ml. It does not involve heating, extraction with organic solvents, daily preparation of reagents, or multiple centrifugations. It is applicable to serum or plasma, including cloudy and jaundiced specimens.

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DIFFERENTIATION OF Fe^{59} AND Cr^{51} IN MIXTURES

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INTRODUCTION

THE methods and advantages of obtaining simultaneous determinations of plasma iron clearance, plasma iron turnover, per cent iron utilization, blood volume, and erythrocyte survival time with the use of Fe^{59} and Cr^{51} simultaneously have been demonstrated.[1] The counting techniques suggested[1, 2] for differentiating between the two radioactive isotopes, Fe^{59} and Cr^{51}, in mixtures depend on the energy discrimination between the 1.3 mev gamma rays from Fe^{59} and the 0.32 mev gamma rays from Cr^{51}. The discrimination is accomplished by means of a gamma ray pulse-height selector or a pulse-height analyzer.

Since only a limited number of institutions are presently equipped with pulse-height discriminators, it appears appropriate to point out that these two isotopes, Fe^{59} and Cr^{51}, in mixtures can be separated readily by means of a simple lead absorber, since the difference in absorption in lead of the gamma rays of these two isotopes is quite marked. This method, while not as elegant as those suggested, has the advantage of simplicity and minimum equipment cost and is quite adequate for the problem. With the provision of the lead absorbers described below any institution equipped with a scintillation detector can carry out the necessary procedures.

MATERIALS

The "lead absorber" method of separating mixtures of Fe^{59} and Cr^{51} has been adapted for two types of scintillation detectors: the standard NaI 1¾- by 2-inch well crystal with a ⅝-inch diameter well and the 1½- by ¾-inch flat crystal commonly used for thyroid "uptake" studies.

Fig. 1 shows the type of wooden holders, lead absorbers, and sample containers required for each type of scintillation detector. The dimensions shown for the accessories for the well counter should fit any standard 1¾-inch by 2-inch well crystal. The dimensions for the accessories for the flat crystal may have to be modified to fit various manufacturers' scintillation detectors. Those shown were adapted for use with a LAX 12 P Scintimeter with lead shield.*

The dimensions of the wooden holders are identical with those of the lead absorbers. The wooden holders provide a means of maintaining a constant geometry and are used to obtain the "no absorber" sample counts discussed below.

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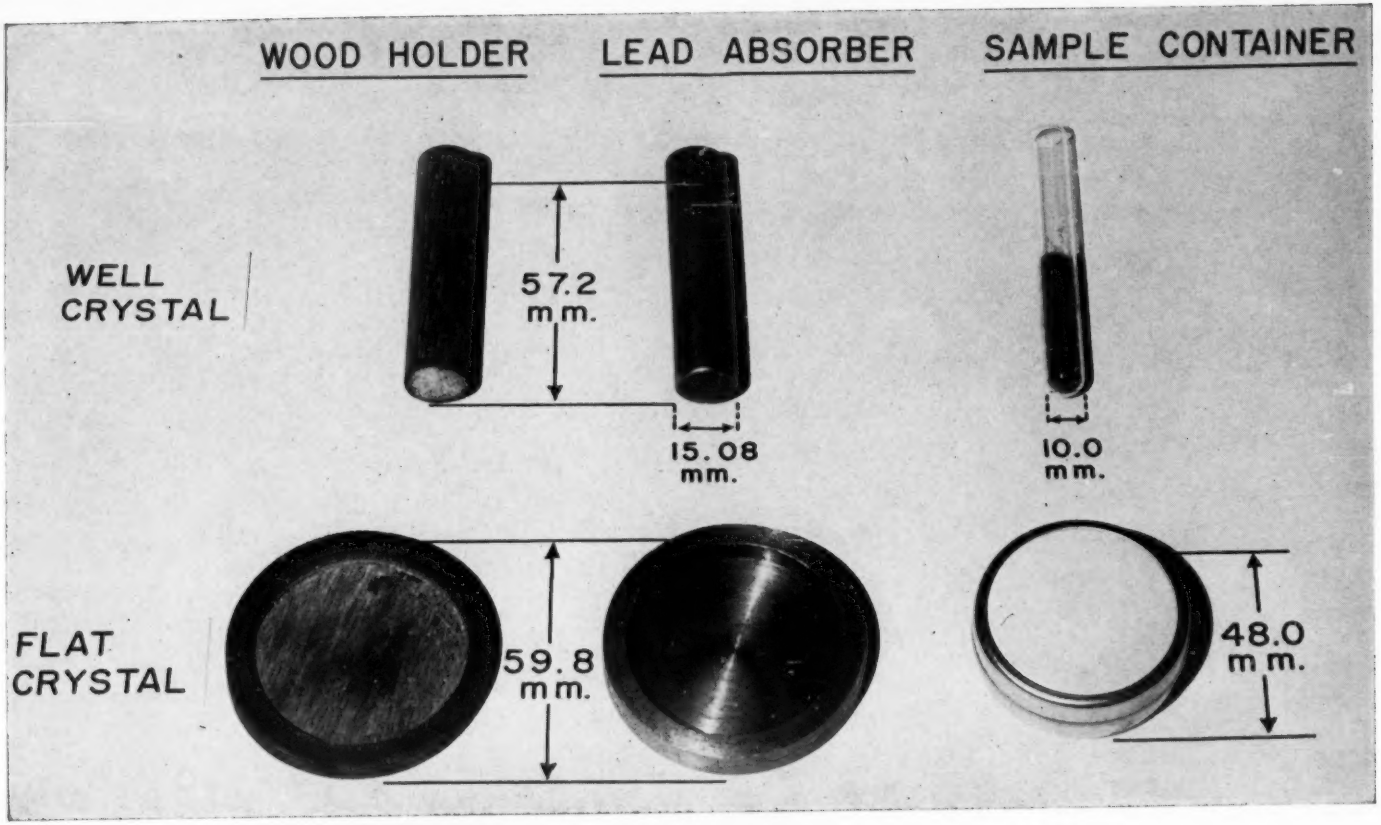

Fig. 1.—Auxiliary equipment required for differentiating Fe^{59} and Cr^{51} in mixtures.

All of the dimensions for the lead absorbers except the thickness of the lead are shown. The well-type absorber has an approximate thickness of 2.38 mm. in all dimensions, the flat-type lead absorber has an approximate thickness of 2.38 mm. only in the area covered by the bottom of the sample container.

The sample containers for the well-type crystal are 10 × 75 mm. culture tubes without lip. The sample containers for the flat-type crystal are Buckeye 1 oz., style No. 100, seamless tin boxes.*

METHOD

The radioactivity of each of the two isotopes Fe^{59} and Cr^{51} in a mixture may be resolved by means of the following equations:

1. Net count (no absorber)† = Fe + Cr
2. Net count (lead absorber) = $K_{Fe}Fe + K_{Cr}Cr$

K_{Fe} and K_{Cr} are constants (fraction of count remaining with lead absorber) and are obtained with samples of Fe^{59} and Cr^{51} prior to the determination of the two separate radioactivities in mixtures.

$$K_{Fe} = \frac{\text{Fe net count (lead absorber)}}{\text{Fe net count (no absorber)†}}$$

$$K_{Cr} = \frac{\text{Cr net count (lead absorber)}}{\text{Cr net count (no absorber)†}}$$

In this laboratory the following values were obtained for K_{Fe} and K_{Cr}.

	WELL CRYSTAL	FLAT CRYSTAL
K_{Fe}	0.82	0.87
K_{Cr}	0.33	0.316

The Fe^{59} and Cr^{51} counts in mixtures of these two isotopes are obtained by substituting the net count observed for the mixture with no absorber in Equation 1, and the net counts observed for the mixture with lead absorber and the K_{Fe} and K_{Cr} values in Equation 2, and solving the two equations for Fe^{59} and Cr^{51} radioactivities.

ILLUSTRATIVE EXAMPLE

If the net count for a mixture of Fe^{59} and Cr^{51} with no absorber equals 1,000 counts per minute and the same sample counted with the lead absorber in the well crystal equals 500 net counts per minute, find the Fe^{59} and Cr^{51} net counts per minute in the sample.

1. 1,000 CPM = Fe^{59} + Cr^{51}
2. 500 CPM = 0.82 Fe^{59} + 0.33 Cr^{51}

These two equations are solved in the usual manner by eliminating one of the two unknowns. In this instance this is accomplished by dividing Equation 2 by 0.82 obtaining:

3. 610 CPM = 1 Fe^{59} + 0.403 Cr^{51}

Subtracting Equation 3 from Equation 1 above.

4. 390 CPM = 0.67 Cr^{51}
5. Cr^{51} = 582 CPM

By difference (1,000 - 582)

6. Fe^{59} = 418 CPM

Results With Known Mixtures of Fe^{59} and Cr^{51}.—Table I shows, for a series of mixtures of Fe^{59} and Cr^{51}, the per cent each component comprises of the total count in the known mixture, and the per cent of each as determined by applying the "lead absorber" method as described for the separation of the two radioactivities.

*The Buckeye Stamping Company, Columbus, Ohio.
†Wood holder.

TABLE I. COMPARISON BETWEEN KNOWN MIXTURES OF Fe^{59} AND Cr^{51} AND THE PER CENT OF EACH AS DETERMINED BY THE "LEAD ABSORBER" METHOD

MIXTURE NO.	PER CENT (KNOWN MIXTURE)		PER CENT (LEAD ABSORBER METHOD)	
	Fe^{59}	Cr^{51}	Fe^{59}	Cr^{51}
1	98.40	1.60	98.62	1.38
2	96.70	3.27	96.27	3.73
3	64.90	35.15	63.07	36.93
4	48.00	52.00	48.84	51.16
5	31.60	68.40	32.74	67.26
6	2.80	97.20	3.50	96.50
7	1.42	98.58	1.51	98.49

DISCUSSION

There are two sources of error contributing to the differences observed in Table I between the per cent of Fe^{59} and Cr^{51} in known mixtures of these two radioactive isotopes and the per cent of each as determined by the "lead absorber" method. First, there are the dilution and pipetting errors introduced during the preparation of the known mixtures and, second, counting errors due to the random nature of the decay process. The counting error can be minimized to any value desired by observing a sufficiently large number of counts. For the data shown in Table I, the counting errors are less than $\pm$ 1.0 per cent.

The differences in the per cent Fe^{59} and Cr^{51} in the known mixtures and those calculated from the "lead absorber" method are less than $\pm$ 5.0 per cent, except in those instances where the Fe^{59} or the Cr^{51} is less than 3.0 per cent of the total count in the mixture. It is probable that the greater differences between the known and calculated values in those instances where one or the other of the two isotopes constitutes less than 3.0 per cent of the total count were largely due to our inability to accurately add a few hundred counts to the "known" mixture. This type of error is not inherent in counting mixtures of Fe^{59} and Cr^{51} in blood samples from patients. One would expect, therefore, that the only source of error would be that due to counting the sample.

Comparison of Well Versus Flat Crystal.—It is of considerable interest to compare the relative efficiencies of the well crystal and the flat crystal for Fe^{59} and Cr^{51}. These are shown in Table II in terms of counts per minute per microcurie.

TABLE II. EFFICIENCY OF WELL VERSUS FLAT CRYSTAL FOR Fe^{59} AND Cr^{51}

ISOTOPE	COUNTS/MINUTE/MICROCURIE	
	WELL CRYSTAL	FLAT CRYSTAL
Fe^{59}	823,000	261,250
Cr^{51}	99,100	19,050

The loss in count for the flat crystal is due largely to the less favorable geometry. The relatively larger loss in count for the Cr^{51} as compared to the Fe^{59} for the flat crystal is due to absorption losses in the aluminum window of the detector. However, for the practical problem of determining the Fe^{59} and

Cr^{51} radioactivities in mixtures in blood samples, the loss in counts for the flat crystal is compensated for by an increase in the volume of the blood sample counted in the tin sample container. Two milliliter volumes are used in test tube sample container for the well crystal and a volume of 10 ml. may be used in the tin sample container for the flat crystal.

SUMMARY

A simple "lead absorber" method has been described whereby any institution equipped with a scintillation detector may determine Fe^{59} and Cr^{51} radioactivities in mixtures.

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ELECTRONIC BED BALANCE; COMMENTS ON ITS USE IN THE CLINICAL ESTIMATION OF CHANGES IN BODY WATER CONTENT AND RATE OF INSENSIBLE WATER LOSS

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THE electronic weighing apparatus described here was developed to make it possible to weigh a patient accurately while in bed. The results obtained are discussed with relation to the clinical problem of estimating changes in body mass or water content and of estimating rate of insensible water loss via skin and lungs.

THE ELECTRONIC BED WEIGHING SYSTEM

As indicated in Fig. 1, the bed frame on which the patient rests is supported at its corners by four Baldwin load-sensing devices, of 45 kilograms capacity each. These devices consist of small metal beams, to each of which a Baldwin SR-4 strain gage is bonded. The latter consists of a fine wire, the resistance of which changes in proportion with strain or deformation. The resistance changes of the four strain gages are integrated by a Baldwin precision indicator, which automatically translates these into weight values. The indicator scale is calibrated in ten gram increments and covers the range of zero to 90 kilograms. Responses are registered to a change in weight of the bed frame of five grams or more.

The accuracy of the apparatus has been tested with 21 known weights, ranging between a minimum 0.5 and a maximum of 47 kilograms. These weights were added to the bed in a random manner on several different days. At least three measurements were carried out with each weight tested.

Statistical analysis of 72 measurements reveals a mean deviation of 0.132 per cent and a standard deviation of 0.178 per cent. This standard deviation is reduced to 0.126 per cent if weighings are carried out in duplicate and to 0.103 per cent if they are performed in triplicate. On the average it takes about one-half minute to make a single measurement. Accordingly it is practicably feasible to take advantage of the increase in accuracy resulting from weighing in duplicate or triplicate.

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An error of ± 1.03 grams per kilogram (triplicate weighing) appears small and of little practical importance when viewed with relation to the changes in body water observed in dehydrated individuals where losses of 100 milliliters and more per kilogram may be encountered. The same may be said for edematous or water-intoxicated patients, where abnormal gains ranging from 50 to several hundred milliliters per kilogram may be seen.

The significance of this error may be viewed also with relation to the clinical estimation of insensible weight or water losses by patients of various sizes. Here, because insensible weight losses per 24 hours may be as small as

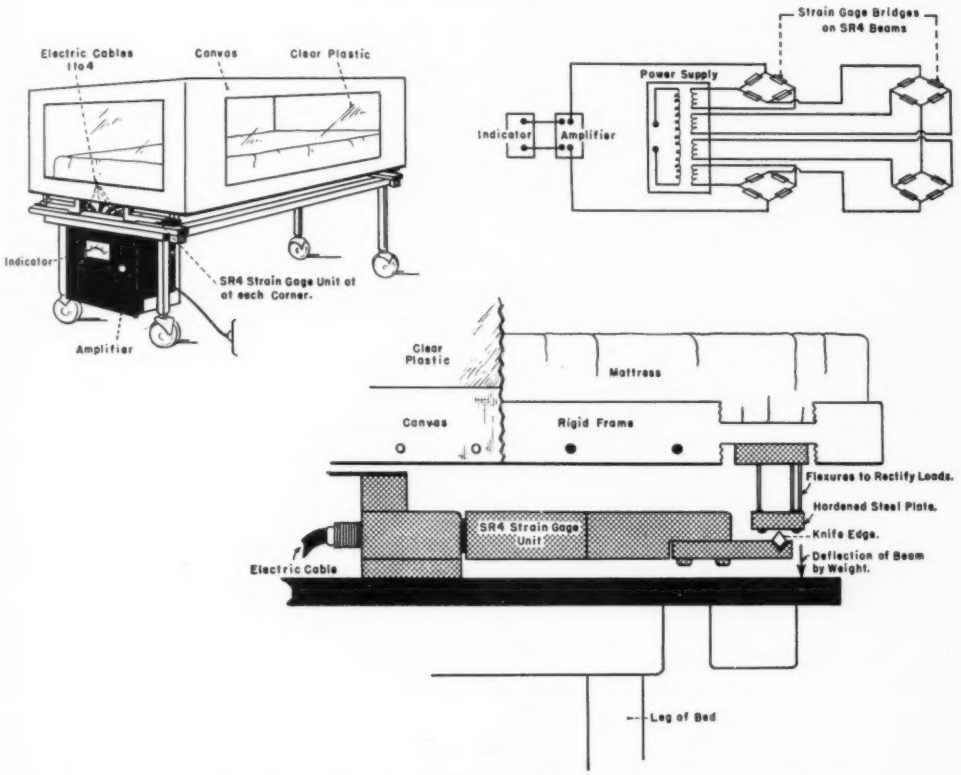

Fig. 1.—Electronic bed-weighing apparatus. Note that the frame holding the mattress is supported at each of its four corners by a beam to which is attached a strain gage sensing element. Not shown is a lever which serves to raise the mattress frame off these beams between weighings.

1.0 per cent and seldom in clinical circumstances exceed 10 per cent of body weight, even small errors in the latter measurement tend to result in relatively large errors in determination of insensible water losses. These relations have been expressed in practical clinical terms in Fig. 2.

In making the estimates plotted in this figure, we assumed in accordance with recent observations[1] that insensible water losses of hospitalized patients amount on the average to approximately 1,200 ml. per square meter (m^2) per day in infants of about 4.5 kilograms or 0.25 m^2 surface area and that as

persons grow larger they decrease steadily to a level of approximately 700 ml. per square meter per day in individuals of 50 kilograms or more (1.5 m^2 or more). Since the rate of insensible water loss will be dependent upon the difference between two weighings, each subject to errors as defined above and separated by a known time interval, the standard deviation of the insensible water loss estimate will be equal to the square root of the sum of the standard deviation squared of each of the two weight estimates. The times needed for individuals to lose sufficient weight so that the standard deviation of the rate estimate as determined by means of single, duplicate, and triplicate weighings, respectively, would equal approximately ± 5 per cent of the insensible loss were then calculated and plotted according to the ordinate scale against body weight per the abscissal scale.

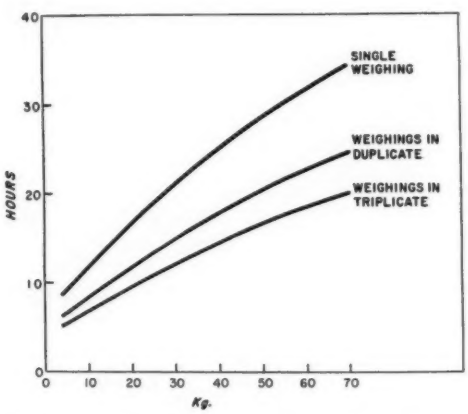

Fig. 2.—Approximate time (ordinate) required to estimate rate of insensible water loss in individuals of various weights (abscissa) with an error of ± 5 per cent when each weighing is done singly (upper curve), in duplicate (middle curve), or in triplicate (lower curve).

The curves indicate that the time required to obtain reasonably representative insensible weight loss values increases as individuals grow larger. Specifically speaking, when weighings are performed in triplicate, it takes from 5.1 (3.5 kilogram infants) to 20.2 hours (70 kilogram adult) to estimate insensible weight loss with an error of ± 5 per cent. These error values are doubled when the interval between weighings is halved and vice versa. These relations reflect the fact that the absolute error in weight measurement increases while insensible water loss per unit of mass declines as individuals become larger.

In making the foregoing calculations, it was assumed that no other errors exist. While this may be true for a few hours during which the patient neither eats nor drinks and has no excreta, it is not true over longer periods where these processes are taking place. Under the latter circumstances, errors incident to weighing of food and fluid taken and urine and stool excreted must be added to those incurred in weighing the patient. It follows that gains in accuracy incident to prolongation of the interval between body weight

measurements beyond a minimum satisfactory number of hours probably will be offset or eliminated by these additional measurement errors. In view of the foregoing, it would appear desirable to use the shortest interval of measurement compatible with reasonably reliable results.

Consideration of the relations outlined above indicates that it will not be easily possible to reduce the error of estimating insensible water loss by improving the accuracy of patient-weighing apparatus beyond that described here. Rather, it would appear that improvement in accuracy should be sought by devising direct means for determining the weight of water leaving the body via skin and lungs.

SUMMARY

This paper describes an easily operated, electronic bed-weighing apparatus. Tests with calibrated loads show that the apparatus measures weight with an average error of ± 0.13 per cent (standard deviation ± 0.178 per cent). The significance of these errors with relation to clinical estimations of change in body mass or water content and of rate of insensible water loss is discussed.

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PRESERVATION OF SHEEP ERYTHROCYTES AND THEIR USE IN A RAPID PLATE TITRATION OF HETEROPHILIC ANTIBODIES IN INFECTIOUS MONONUCLEOSIS

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WITH THE TECHNICAL ASSISTANCE OF BONNIE FINGERHUT, A.B.

THE titration of heterophilic agglutinins for sheep erythrocytes is a widely used procedure in the laboratory diagnosis of infectious mononucleosis. Procedures for tube dilution agglutination titration, as well as for differential adsorption tests with guinea pig kidney and beef erythrocytes, have been well defined.[1-4] Various attempts have been made to decrease the time for performance[5-7]; however, such proposals involve the rapid reading of tube agglutination titrations by centrifugation using freshly washed cells, or they have been strictly qualitative tests. Stein[8] reported on the use of erythrocytes preserved in a dextrose-gelatin-Veronal solution; however, the life of such a preparation was somewhat over one month.

In the course of other experiments in this laboratory it became evident that we could preserve sheep erythrocytes for long periods of time. The advantage of a stable preserved sheep erythrocyte antigen (PSE) is obvious, both from the standpoint of time involved in preparation of erythrocyte suspensions and of decreasing errors arising from variation in the susceptibility of erythrocytes from different sheep to heterophilic agglutination.[9] The possible application of such preserved erythrocytes was therefore studied with respect to the heterophilic agglutination reaction.

MATERIALS AND METHODS

Diluent throughout consisted of 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M. KH_2PO_4 in distilled water, the pH being 7.2 ± 0.05. When needed, diluent was sterilized by autoclaving. Rapid agglutination tests were performed on a large glass plate marked with circles of 18 mm. internal diameter. It was found advantageous to use a flat plate, since readings were more difficult on concavity depressions. All tube agglutination titrations were performed in 13 × 100 mm. Pyrex tubes as previously described.[2] Tubes were observed after incubation in the 37° C. water bath for two hours. Freshly washed sheep erythrocytes previously collected in Alsever's solution were used. Titers were expressed as the highest dilution of serum showing definite agglutination.

Preparation of PSE.—Whole sheep blood was kept refrigerated in equal volumes of sterile Alsever's solution. Forty milliliters of 50 per cent formalin-diluent (equal volumes of formalin and twice-concentrated diluent above) were rapidly mixed with 100 ml. whole sheep blood in Alsever's solution. The formalinized blood was placed in a 37° C. water bath for 1½ to 2 hours with frequent mixing. It was found that freshly defibrinated sheep

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blood diluted with an equal volume of diluent could be used in place of sheep blood in Alsever's solution. Aseptic precautions were maintained. The formalinized blood changed color gradually to dark brown, which usually required over an hour. It was essential that incubation proceed until the red color had changed to a distinct brown. The blood was then centrifuged, and the cells were packed and washed 6 times, in at least 50 volumes of diluent each time, care being taken to remove and discard by aspiration the top thin, light-colored coat after the first centrifugation. The supernatant was usually clear after the second wash. The washed and packed erythrocytes were then resuspended in diluent containing 0.3 per cent formalin to approximately 10 per cent by volume. Cell volume was more accurately adjusted to 4 per cent by centrifugation of 5.0 ml. of the 10 per cent suspension in a Kolmer or vaccine tube and determination of the volume of packed cells. As an additional precaution against contamination during use of the cells after preparation, 0.25 ml. of a dye mixture (1 part of 1 per cent aqueous crystal violet plus 2 parts 1 per cent aqueous brilliant green) was added and mixed with each 100 ml. of 4 per cent suspension. Addition of dye also facilitated reading. This antigen was dispensed in sterile screw cap dropper-vials and refrigerated. Droppers were selected to deliver 0.025 ml. per drop (39 to 41 drops per milliliter). Each batch of PSE was checked for sensitivity against at least five heterophilic antisera of known tube agglutination titers as well as negative serum.

Procedure for Rapid Plate Titration of Human Sera.—The procedure followed was similar to that used in rapid plate bacterial agglutination tests.[10] Human serum was separated from clotted blood in the usual manner. Inactivation was not necessary. Serum, undiluted and diluted 10^{-1} and 10^{-2}, was pipetted onto appropriate rings on a glass plate, and 1 drop of PSE was immediately added to each ring, as indicated in Table I. Serum was pipetted with Bangs pipettes or 0.2 ml. pipettes graduated in 0.01 ml. divisions, each serum dilution mixed with a separate applicator stick from right to left, rotated for 1 to 2 minutes by hand and read macroscopically over a light in the usual manner of reading rapid plate bacterial agglutination tests.

To avoid dilution errors in interpreting titers of adsorbed sera, we carried out adsorptions on sedimented boiled beef cells and guinea pig kidney tissue centrifuged from saline suspensions. Thus the serum was not diluted during the absorption step, and adsorbed serum titers then corresponded to those indicated for nonadsorbed sera in Table I.

TABLE I. RAPID TITRATION OF HUMAN SERUM

	CIRCLE			
	1	2	3	4
SERUM, ML.	0.08	0.04	0.02	0.01
PSE, 0.025 ML./DROP	1 DROP	1 DROP	1 DROP	1 DROP
Series 1. Serum undiluted	20*	40	80	160
Series 2. Serum 10^{-1}	200	400	800	1,600
Series 3. Serum 10^{-2}	2,000	4,000	8,000	16,000
Series 4. Negative serum or diluent control				

*Dilutions expressed as reciprocals corresponding to dilutions obtained in tube dilution method.

Each serum was run simultaneously with a diluent or negative serum control. Titer of the serum was expressed as the highest corresponding dilution showing definite clumping. Diluent or negative serum controls should always be negative (fine suspensions of cells, smoothly dispersed).

RESULTS

General Observations.—Preliminary observations demonstrated that in the preservation of the erythrocytes, a final concentration of over 10 per cent

formalin was required. Less than 10 per cent caused considerable lysis. Preservation in the presence of 14 to 20 per cent formalin was accomplished at refrigerator temperatures, but the process required several days. If the erythrocytes were held in 14 per cent formalin for less than 1½ hours at 37° C., the preserved cells showed evidence of lysis within a few days in the refrigerator. Microscopically, in both stained and diluent preparations, the preserved erythrocytes appeared normal with the hemoglobin staining well; it was estimated that less than 10 per cent of the erythrocytes showed evidence of crenation. The addition of formalin-diluent to whole blood rather than washed erythrocytes seemed advantageous. When washed erythrocytes were used, as reported by Flick[12] with human cells, considerable lysis occurred. Furthermore, PSE showed considerable tendency to autoagglutinate when washed erythrocytes were exposed to final concentrations of formalin approaching 50 per cent.

PSE was found to be stable for several months, both from the standpoint of hemolysis and reactivity with antisera. Stable suspensions of PSE have been consistently prepared and maintained for a minimum of ten months in the refrigerator (the longest period checked). Attempts to determine stability at room temperature (25 to 28° C.) have shown no loss of stability within one month. PSE showed no evidence of lysis within several hours in distilled water, nor did suspensions show evidences of lysis when sensitized by two units of sheep erythrocyte amboceptor in the presence of six exact units of complement determined in the usual fashion.

Standardization.—A study of the optimal concentration of PSE revealed that much less than 4 per cent suspension was too light to be easily read and too sensitive in the sense that the suspension seemed too granular. Much greater than 4 per cent suspension was too heavy for easy reading and proved insufficiently sensitive with respect to the assigned serum dilutions in Table I when titrated by the rapid plate technique against known heterophilic antisera. A 4 per cent suspension was consistently found to yield end points in the rapid plate procedure in close agreement with tube agglutination titers, when serum dilutions were assigned to PSE-serum mixtures, as indicated in Table I.

Comparison of Rapid Plate With Tube Agglutination Technique.—A comparison of titers obtained by the rapid plate with those obtained by the tube agglutination technique against fifteen infectious mononucleosis human sera is shown in Table II. It may be seen that good agreement is obtained between both procedures. All 15 sera were from individuals who were clinically typical of infectious mononucleosis, in addition to the results of their heterophilic antibody titration and differential adsorption tests being typical for infectious mononucleosis. The greatest disagreement seen was one tube dilution, which discrepancy is considered to be within the limits of experimental error in the use of either one of the two techniques. The differences between titers, for example of 1:800 versus 1:640, are merely differences in the dilution sequence represented by each procedure. Similar agreement between both procedures may be noted with the first 30 sera in Table II which were from patients who were not considered clinically typical of infectious mononucleosis.

A comparison of the tube and rapid-plate procedures on the 15 positive sera adsorbed with guinea pig kidney tissue and boiled beef cells showed results comparable in agreement with those in Table II. For example, one unadsorbed serum having a rapid titer of 1:2,000 and a tube titer of 1:2,560 showed a rapid titer of 1:1,600 and a tube titer of 1:1,280 after adsorption with guinea pig kidney, and titers of <1:20 with both procedures after adsorption with boiled beef cells.

The possibility was also considered that PSE could be used to titrate rapidly antisera produced in the rabbit against sheep erythrocytes. Upon titrating several batches of PSE (4 per cent suspension) against two antisheep erythrocyte rabbit sera, using the rapid-plate technique, we obtained good correlation between rapid-plate and tube dilution titers. Results were similar to those presented in Table II.

TABLE II. COMPARISON OF RAPID PLATE WITH TUBE AGGLUTINATION TITERS

NUMBER OF HUMAN SERA	RAPID TITER*	TUBE TITER*
22	< 20	< 20
1	20	< 20
1	20	20
1	40	20
2	40	40
1	40	80
2	80	80
2†	200	320
1†	400	640
3†	800	640
1†	800	1,280
2†	1,600	1,280
2†	2,000	2,560
2†	4,000	5,120
2†	8,000	10,240

*Titers expressed as reciprocals.

†Results of differential adsorption studies on these sera were typical for infectious mononucleosis; i.e., the antibodies were completely adsorbed on boiled beef erythrocytes and not on guinea pig kidney.

Use of PSE in Tube Agglutination Procedures.—After it became evident that PSE could be used in the rapid-plate technique to obtain results comparable with those by the usual tube method, the possible use of PSE to replace freshly washed erythrocytes in tube dilution heterophilic agglutination tests was considered. PSE was studied in concentrations of 0.25 per cent and 0.5 per cent, but no evidence of agglutination with known infectious mononucleosis sera occurred when read by gently flicking the tube. Hemagglutination was evident, however, when read by the pattern method.[11] Even when positive patterns occurred, upon flicking of the tube the cells resuspended smoothly. Furthermore, the addition of 30 per cent bovine albumin or the subsequent addition of anti-human globulin rabbit serum to the washed red blood cells did not induce agglutination. Occasionally, with very high titer heterophilic sera, weak agglutination was observed by flicking of the tube, but only in low serum dilutions.

DISCUSSION

In the experience of the authors, the presently described rapid-plate procedure using PSE as the antigen provides an extremely rapid method for titrating heterophilic antibodies in infectious mononucleosis. Specificity and sensitivity with both unadsorbed and adsorbed human sera correlates well with contemporary tube agglutination procedures employing fresh erythrocytes, and the stability of PSE offers the distinct advantages of employing constant cellular antigens and eliminating the daily washing of erythrocytes for this test. Furthermore, resistance of PSE to lysis eliminates the necessity of inactivating sera before titrating.

Since the above conclusions are based upon findings with a relatively small number of infectious mononucleosis sera, it is felt that the results justify a more extensive trial by others. The significance of the above preservation of sheep erythrocytes would seem to extend beyond its application in the heterophile agglutination test for infectious mononucleosis, since erythrocyte suspensions are being widely used to adsorb various microbial antigens in hemagglutination and hemolytic procedures. Further work along this line is in progress.

SUMMARY

The preparation of preserved sheep erythrocytes (PSE) and their use in a rapid-plate hemagglutination procedure for the titration of sheep erythrocyte antibodies are described. Such preserved cells were microscopically typical in appearance and were not subject to lysis either in distilled water or by sensitization with amboceptor in the presence of complement. PSE preparations were stable for long periods of time. The rapid-plate technique with PSE showed good correlation with the tube agglutination test using freshly washed sheep erythrocytes during titration of heterophilic antibodies in both unadsorbed and adsorbed infectious mononucleosis sera, as well as during titration of antisheep erythrocyte rabbit sera and sheep erythrocyte antibodies in sera of humans without infectious mononucleosis. In tube agglutination tests with heterophilic antisera from infectious mononucleosis, PSE consistently showed agglutination only when read by the pattern method.

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THE DETERMINATION OF TOTAL URINARY OXALATE

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INTRODUCTION

THE widespread occurrence of oxalate in urinary calculi has focused attention upon the excretion of oxalic acid in the urine. For this reason the need for an accurate method for the routine determination of urinary oxalate is apparent. Such a method would permit comparison of the oxalate excretion patterns of normal individuals with those of chronic stone formers.

Methods existing in the literature are based either upon direct precipitation of oxalate from the urine[1] or upon a preliminary extraction of the acidified urine with ether, followed by determination of oxalate in the extract.[2-5] The direct precipitation method is subject to interference by magnesium, phosphate, and sulfate. Some investigators[2, 3] have isolated oxalates by elaborate distillation techniques. Although yielding excellent results, this technique does not readily lend itself to the routine handling of a large number of samples. Those methods depending upon precipitation of oxalate as the calcium salt from the ether extract of urine have usually failed to give quantitative recoveries of added oxalate, Powers and Levatin[5] reporting recoveries ranging from 89 to 93 per cent, depending upon the amount of oxalate added. These low recovery values are apparently caused by incomplete extraction or by destruction of the oxalic acid present.

Bau[1] and Powers and Levatin[5] have pointed out the case of hydrolysis of oxaluric acid to oxalic acid and urea under the conditions used for extraction of the oxalic acid from urine. These latter investigators have stressed the point that, unless a preliminary hydrolysis of oxaluric acid is carried out, somewhat variable results are obtained on analysis of urine for oxalates. For this reason Powers and Levatin indicate that the ether extraction procedure at best can only give total oxalate values for urine, that is, oxalic acid plus oxaluric acid.

In view of the apparent difficulties associated with the direct determination of oxalate in urine, it was decided to investigate more thoroughly the method of Powers and Levatin utilizing the ether extraction procedure. Specific points

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for consideration were time required for complete extraction, purity of the extracting solvent, and conditions for precipitation of calcium oxalate from the extract.

MATERIALS AND METHODS

Apparatus.—Liquid-liquid extractor, as described by Kyker, Webb, and Andrews,[6] modified for use with 25 or 50 ml. samples and adapted to standard taper glassware (Fig. 1).

Suction device as described by Powers and Levatin.[5]

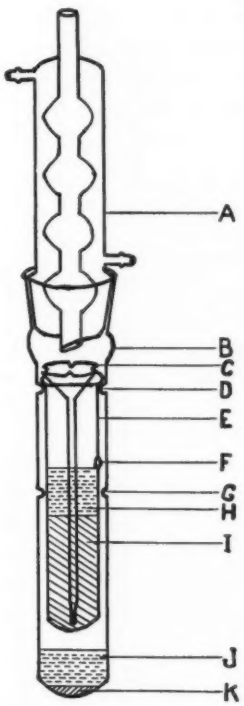

Fig. 1.—The extraction apparatus. The parts and assembly of the extractor for the extraction of the urine sample are described as follows: *A*, Allihn condenser S. T. 45/50 joint; *B*, outer tube of extraction apparatus with outer member of S. T. 45/50 joint at top, over-all length 430 mm., outside diameter, 41 mm.; *C*, funnel, diameter 35 mm. and length 290 mm., with four indentations in rim spaced 90° apart; *D* and *G*, punch marks 245 mm. and 200 mm., respectively, from bottom of outer tube, for support of sample tube *E*; *E*, inner tube of extraction apparatus, 29 × 200 mm. test tube with reinforced lip, and a hole 8 mm. in diameter in side of tube located 80 mm. from top; *F*, overflow hole for ether, 8 mm. in diameter; *H*, ether layer; *I*, acidified sample of urine; *J*, ether reservoir, 25 to 30 ml.; *K*, water layer, 2 to 3 ml.

Reagents: Hydrochloric acid 2 N

Ether, peroxide free

Alcohol, 95 per cent

Bromthymol blue, 0.2 per cent

Nitrogen

Calcium chloride, saturated solution

Acetic acid, glacial

Alcoholic buffer prepared according to Powers and Levatin.[5] To 60 ml. of 95 per cent alcohol, add 10 ml. of 2 per cent acetic acid and 20 ml. of water. Adjust pH to 4.0 by the dropwise addition of concentrated ammonium hydroxide.

Ammonium hydroxide, concentrated

Sulfuric acid, 1 N

Manganese sulfate, 10 per cent

Potassium iodide, 10 per cent

Potassium permanganate, 0.1 N

Sodium thiosulfate, 0.1 N

Starch solution, 1 per cent indicator grade

Standard potassium permanganate 0.01 N; prepare fresh daily by dilution of 0.1 N potassium permanganate, standardize just prior to use.

Standard sodium thiosulfate, 0.01 N; prepare fresh daily by dilution of 0.1 N sodium thiosulfate. Standardize just prior to use.

Procedure.—In the inner tube of the extraction apparatus are placed 25 ml. of urine and 25 ml. of 2 N hydrochloric acid and the contents are mixed by swirling. The tube is then heated for 45 minutes on a water bath heated to boiling without actually boiling, in order to hydrolyze the oxaluric acid present. The tube and contents are cooled under the tap and the acidified urine is overlaid with peroxide-free ether up to the overflow outlet. Approximately 2 ml. of distilled water, 2 glass beads, and 25 to 30 ml. of peroxide-free ether are placed in the outer tube of the extraction apparatus. The extraction apparatus is now assembled (Fig. 1), and the extraction is carried out for 18 hours over an electric heater capable of producing sufficient heat for the gentle boiling of the ether. Care should be taken to prevent bumping as the ether begins to boil. After extraction is complete, the inner tube is removed from the extraction apparatus after its outer wall is washed down with a few milliliters of ether. The ether overlaying the aqueous extract in the outer tube is evaporated on a water bath at 50° to 60° C. with stirring, care being taken to prevent loss of material by bumping. The aqueous layer remaining in the outer tube is transferred to a 50 ml. centrifuge tube by means of the suction device. The outer tube is washed three times with 2 ml. aliquots of 95 per cent ethanol and the washings are added to the contents of the centrifuge tube.

The contents of the centrifuge tube are adjusted to a slight alkaline reaction to bromthymol blue by the dropwise addition of concentrated ammonium hydroxide, while under constant stirring with a nitrogen bubbler. After the addition of three drops of saturated calcium chloride, 1.5 ml. of glacial acetic acid is added to give a final pH of 4.0 to 4.5. The solution is then overlaid with 2 ml. of the alcoholic buffer and allowed to stand at room temperature for 20 minutes. The layer of alcoholic buffer tends to prevent crawling of the precipitated calcium oxalate. After heating on a hot water bath (80 to 90° C.), the centrifuge tube is allowed to stand at room temperature for 3 to 6 hours to permit complete precipitation of calcium oxalate to take place. The sample is then centrifuged at 2,000 r.p.m. for 15 minutes and the supernatant fluid carefully decanted. The tube is allowed to drain for 15 minutes and the well-packed precipitate is broken up by means of a finely drawn stirring rod and washed twice with 2 ml. portions of the alcoholic buffer solution, care being taken to wash down any precipitate clinging to the sides of the tube. After each wash, centrifugation and decantation are carried out as previously described.

After the final wash, the tube is allowed to drain and then is dried on the hot water bath (80 to 90° C.) for one hour. The *dried* precipitate is then broken up and dissolved in 5 ml. of 1 N sulfuric acid. While stirring the resulting solution in the centrifuge tube by means of a nitrogen bubbler, add 0.5 ml. of 10 per cent manganese sulfate, followed by

an excess, usually no more than 5 ml., of standard 0.01 N potassium permanganate. After oxidation of the oxalic acid is complete, 0.5 ml. of 10 per cent potassium iodide is added and the resulting solution titrated with standard 0.01 N sodium thiosulfate from a burette calibrated to read in 0.01 ml. Starch is used as an indicator.

Calculations:

(milliequivalents of permanganate − milliequivalents of thiosulfate) × 45 × 24-hour volume of urine/25 = mg. anhydrous oxalic acid/24 hours.

RESULTS

Extraction Time.—The time required for complete extraction of acidified aqueous solutions containing 3.60 mg. of anhydrous oxalic acid was determined by the procedure outlined above. Two series of determinations in quadruplicate gave recoveries in all cases ranging from 100.0 to 100.2 per cent for an 18-hour extraction period. The time required for complete extraction of urine was also determined. Recoveries of 3.60 mg. of oxalate added to 50 ml. of acidified,

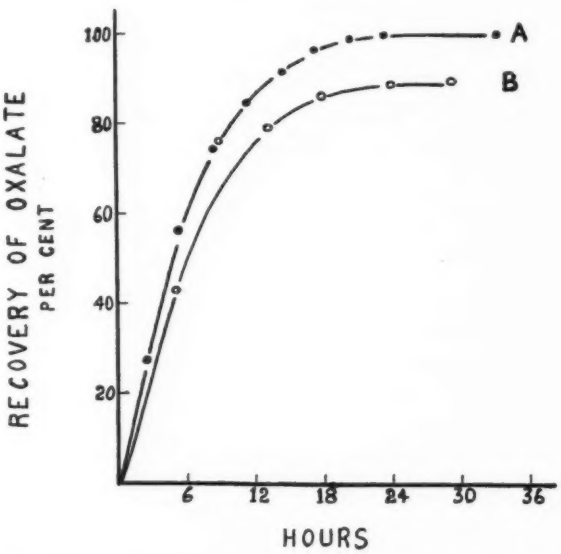

Fig. 2.—Extraction time required for complete recovery of oxalate from urine. Curve *A*, recovery of oxalate when peroxide-free ether was used. Curve *B*, recovery of oxalate when peroxide-containing ether was used.

hydrolyzed urine were made, stopping the extractions at intervals of 3 hours and continuing through a period of 33 hours, and analyzing the extract at each interval for oxalate. At the same time the effect of peroxides in the extracting ether was investigated. A similar series of recoveries on the same urine as used above was carried out with use of ether known to contain peroxides as the extracting medium. The results of these experiments are shown in Fig. 2. Curve *A* shows the results obtained with peroxide-free ether and Curve *B* those obtained with peroxide-containing ether. When peroxide-containing ether was used, the recovery of oxalate was approximately 15 per cent lower than was the case when peroxide-free ether was used.

Precipitation of Calcium Oxalate.—The pH at which calcium oxalate is precipitated from the extract of the urine is of considerable importance. At pH 6 and above, amorphous calcium phosphates coprecipitate with calcium oxalate, since small amounts of phosphate are extracted from the urine by the procedure used. In addition, whenever precipitation is carried out at pH 6.0 or above, some reducing substance or substances are apparently adsorbed on the calcium phosphate formed. These substances are likely to be reducing ketosteroids since they are known to be extracted by the procedure used. To investigate the possibility of eliminating this interference, we carried out a series of extractions as described above. Duplicate extractions were stopped at intervals of 3 to 6 hours over a period of time extending to 28 hours. One set of extractions was precipitated at pH 6.8 and the other at pH 4.0 to 4.5, and the precipitate was analyzed for oxalate by the method described above. Table I shows that at the time when recovery of oxalate was virtually complete

TABLE I. RECOVERY OF OXALATE FROM URINE

EXTRACTION TIME (HOURS)	OXALATE RECOVERED	
	PRECIPITATION AT pH 4.0 (PER CENT)	PRECIPITATION AT pH 6.8 (PER CENT)
3	33.7	41.0
6	76.3	72.4
9	87.3	86.2
12	94.8	94.1
15	96.5	99.3
18	98.3	109.0
21	99.2	114.0
24	99.8	121.0
33	99.8	133.0

(precipitation at pH 4.0 to 4.5) the values obtained by precipitation at pH 6.8 were still increasing. The values obtained by precipitation at pH 6.8 were 9 per cent high at 18 hours extracting time and continued to increase, being 33 per cent high at 28 hours.

The Oxalate Content of Human Urine.—Twenty-four-hour excretions of total urinary oxalates were determined on human urines by the procedure described. Table II shows the results obtained by this method as compared with those obtained by other investigators.

TABLE II. THE OXALATE CONTENT OF HUMAN URINE

1. PRESENT METHOD (MG./24 HR.)	2. POWERS AND LEVATIN[5] (MG./24 HR.)	3. BONIFAZ AND MÜLLER[2] (MG./24 HR.)	4. BAU[1] (MG./1)	5. SALKOWSKI[4] (MG./24 HR.)
21.9	22.1	24.5	13.9	22.0
28.5	22.4	34.0	12.0	35.0
30.4	25.1	35.0	17.0	68.3
36.7	27.7	35.0	18.0	70.0
40.5	35.8	40.0		90.0

DISCUSSION

From the data presented it appears that the low recovery values reported by Powers and Levatin are apparently caused by incomplete extraction, since these investigators used a 6-hour extraction time as compared to the 18-hour extraction period found to be necessary in the present method for complete extraction of oxalic acid from the urine. In addition, the present method has shown the necessity for using peroxide-free ether as the extracting medium, since the peroxides present in ordinary commercial ether have been shown to be capable of oxidizing part of the oxalate present in urine. The study on the conditions for precipitation of calcium oxalate led to the finding that reducing substances other than oxalate are adsorbed on the calcium phosphate which coprecipitates with calcium oxalate at pH's of 6 or greater. These reducing substances are capable of reacting with permanganate and thus give high results if not eliminated. They are believed to be reducing ketosteroids. On the other hand, if precipitation is carried out at pH 4.0 to 4.5, no interference by these substances is evident. Although a longer time is required for precipitation of calcium oxalate at pH 4.0 as compared with pH 6.0 or greater, the use of the lower pH avoids the danger of contamination by these unknown reducing substances.

Twenty-four-hour excretions of urinary oxalates have been determined on some 20 human urines and the values have ranged between 20 and 40 mg. per 24 hours. These values compare favorably with those reported by Bonifaz and Müller[2] (25 to 40 mg. per 24 hours) and by Powers and Levatin[5] (20 to 40 mg. per 24 hours). Recoveries of added oxalate from urine by the present procedure have ranged from 98 to 100 per cent as compared with the 89 to 93 per cent reported by Powers and Levatin.

SUMMARY

The method of Powers and Levatin for the determination of total urinary oxalate has been thoroughly investigated.

An extraction time of eighteen hours has been found to be necessary for complete recovery of urinary oxalates by ether extraction. The presence of peroxides in the ether used for extraction destroy part of the oxalate present, making it necessary to carry out extraction with peroxide-free ether.

The pH for precipitation of the extracted oxalate should be in the range pH 4.0 to 4.5 in order to avoid contamination by unknown reducing substances which are adsorbed on the calcium phosphate which coprecipitates at pH 6.0 or higher.

The twenty-four-hour excretion of oxalate in the urine of some twenty normal human subjects has been found to range from 20 to 40 mg. per 24 hours.

Recoveries of added oxalate from urine have ranged from 98 to 101 per cent as compared to the 89 to 93 per cent reported by Powers and Levatin.

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A SIMPLE ULTRAVIOLET SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF PROTEIN

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MEASUREMENT of light absorption at 280 mμ, the approximate position of an absorption maximum for proteins, has been used in the quantitative estimation of protein concentrations.[1] The present report concerns the development of a new spectrophotometric procedure for protein determinations. The use of absorption measurements at shorter wave lengths has permitted the attainment of greater sensitivity, accuracy, and specificity than was possible with methods previously described.

PRINCIPLES INVOLVED IN THE DEVELOPMENT OF THIS METHOD

The absorption maximum exhibited by proteins in the region of 280 mμ is caused by the aromatic amino acids. The intensity of absorption at this wave length depends on the proportions of these amino acids in the protein. Proteins differ accordingly among themselves to a considerable extent in their absorptions in this spectral region.[2] If even small amounts of nucleic acids or nucleotides are present, they interfere seriously with determination of protein by absorption measurements at 280 mμ.

In the spectral range between 200 mμ and 250 mμ, the absorption of proteins increases rapidly with decreasing wave length, reaching intensities at the shorter wave lengths many times greater than that at 280 mμ.[2, 3] The intense absorption at these low wave lengths is due principally to the peptide bonds. Proteins differ among themselves relatively little in their absorption in this spectral region. They differ even less in the slopes of their absorption curves. In the development of this method it was found that the slopes of the absorption curves for human serum albumin and globulin differ so little that the same factor can be used for the calculation of their concentrations without significant error.

In some of the biologic materials in which it is most frequently desired to measure protein, notably plasma, the concentration of protein is so high relative to interfering materials that ultraviolet spectrophotometry permits an essentially specific measure of protein concentration. In the method to be described here, protein concentrations are derived from the difference between the absorbances at 215 mμ and at 225 mμ. The use of the difference rather

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than the absorbance at a single wave length minimizes the error from non-protein constituents of plasma. In ultrafiltrates from several samples of dog and human plasma, the differences in absorbance across the wave length range used have been found so small as to represent a negligible error in the estimation of protein. A concentration of urea far above uremic levels would not interfere significantly. Still greater sensitivity could have been obtained by use of shorter wave lengths, but increased sensitivity is not needed. Below 215 mμ, difficulties may be encountered in obtaining sufficient intensity of light, and the error from scattered light becomes more serious. At 215 mμ it was found that protein solutions conformed closely to Beer's Law so long as the absorbance was less than 2.0. It was inferred that under these conditions scattered light was not contributing a significant error.

Saline rather than distilled water is used as a diluent in order to prevent the precipitation of globulins. The precipitation of globulin in distilled water introduces appreciable error only if the globulin concentration in plasma is abnormally high. Since the absorption of protein in the spectral region used is practically independent of pH over the range of 4 to 8, it is unnecessary to use a buffer as diluent.

PROCEDURE

The protein-containing material is diluted to an appropriate degree with a 9 Gm. per L. solution of NaCl. A Beckman Model DU ultraviolet spectrophotometer is used for the absorption measurements. The absorbance is measured at 215 mμ and at 225 mμ, the saline diluent solution being used as the blank. If the absorbance at 215 mμ exceeds 1.5, the protein solution is further diluted. The absorbance at 225 mμ is subtracted from that at 215 mμ. This difference multiplied by 144 gives the protein concentration in the diluted solution expressed in micrograms per milliliter.*

A dilution of about 1:1,000 is suitable for the determination of total protein in normal plasma. Albumin in plasma is determined by the use of Kingsley's[4] procedure for globulin precipitation.† To 15 ml. of a 225 Gm. per L. solution of Na_2SO_4 in a glass-stoppered tube is added 1 ml. of plasma. After the contents of the tube are mixed by inversion of the tube, 6 ml. of U. S. P. anesthetic ether is added. The tube is shaken and centrifuged. A sample of the clear albumin solution is withdrawn by pipette from beneath the precipitated globulin layer. This is diluted 1:50 with saline and the albumin is determined spectrophotometrically. The globulin concentration is calculated by difference.

A dilution of 1:10 is appropriate for normal cerebrospinal fluid. Higher dilution is necessary if the protein concentration is abnormally high.

RESULTS

The ultraviolet spectrophotometric method has been compared with other methods for the determination of protein in several samples of normal and abnormal human plasma and cerebrospinal fluid and in one sample of pleural

*This factor was found to be identical on two different Beckman DU spectrophotometers. The accuracy of the absorbance calibration of the Beckman instruments is of such an order that restandardization of individual instruments should be unnecessary for an analytic method of this nature. However the factor can be redetermined by use of solutions prepared from crystalline bovine or human serum albumin carefully dried and weighed so as to avoid contamination with moisture or by use of solutions in which the protein concentration has been determined by the Kjeldahl method. Unless the determination of the factor is carried out in such a way as to insure a high degree of precision, it is likely to involve an error greater than that arising from assumption of reproducible absorbance calibrations among different Beckman spectrophotometers.

†Fractionation of proteins with $(NH_4)_2SO_4$ is also permissible in the spectrophotometric method. Because of the ultraviolet absorption of the sulfite ion, Na_2SO_3 cannot be used for globulin precipitation.

fluid. The macro-Kjeldahl method was used for plasma and pleural fluid. Correction was made for nonprotein nitrogen in plasma. The biuret method of Weichselbaum[5] was used for cerebrospinal fluid since the amount of protein was too small to determine with the Kjeldahl method. The results are shown in Tables I and II. The agreement between the Kjeldahl and the spectrophotometric methods for plasma and pleural fluid is quite close. In spinal fluid the agreement between the biuret and the spectrophotometric methods is not always so close, but the deviations scarcely exceed the limits of reproducibility of the biuret method.

TABLE I. COMPARISON OF THE ULTRAVIOLET SPECTROPHOTOMETRIC METHOD WITH THE KJELDAHL METHOD FOR DETERMINATION OF PROTEIN IN TEN SAMPLES OF HUMAN PLASMA AND ONE SAMPLE OF HUMAN PLEURAL FLUID. THE VALUES SHOWN ARE AVERAGES OF DUPLICATE DETERMINATIONS WITH EACH METHOD

MATERIAL	TOTAL PROTEIN (GM. PER 100 ML.)		ALBUMIN (GM. PER 100 ML.)	
	KJELDAHL	ULTRAVIOLET	KJELDAHL	ULTRAVIOLET
Plasma 1	3.84	3.63	2.27	2.33
Plasma 2	6.50	6.53	4.38	4.36
Plasma 3	7.52	7.66	5.38	5.33
Plasma 4	6.78	6.62	4.75	4.79
Plasma 5	6.72	6.70	4.84	4.75
Plasma 6	7.84	7.71	4.53	4.60
Plasma 7	5.66	5.52	3.69	3.67
Plasma 8	6.20	6.26	3.59	3.78
Plasma 9	6.76	7.16	3.98	3.71
Plasma 10	10.68	10.20	2.14	2.19
Pleural fluid	1.40	1.43		

TABLE II. COMPARISON OF THE ULTRAVIOLET SPECTROPHOTOMETRIC METHOD WITH THE BIURET METHOD FOR DETERMINATION OF PROTEIN IN FIVE SAMPLES OF HUMAN CEREBROSPINAL FLUID

SAMPLE	TOTAL PROTEIN (MG. PER 100 ML.)	
	BIURET	ULTRAVIOLET
1	44	48
2	28	36
3	215	204
4	31	39
5	33	43

The values shown are averages of duplicate determinations with each method.

It was found that urine normally contains interfering materials that make this method inapplicable to the determination of protein in urine.

DISCUSSION

This spectrophotometric method has great advantages over such procedures as the Kjeldahl method in simplicity, rapidity, and sensitivity. The likelihood of error is minimal in a method requiring so few steps. Accurate measurement of protein is possible in concentrations as low as 5 mcg. per milliliter. This degree of sensitivity would permit the application of the method in procedures in which it is desired to measure protein in very minute volumes of biologic materials.

This method has been found particularly useful in this laboratory for investigations in which very accurate determinations of plasma protein are required. No conditions have as yet been encountered in which there was evidence of interference from nonproteinaceous materials. The intensity of absorption of protein is so great and the concentration so high that it seems unlikely that any exogenous substance or any substance produced endogenously in pathologic conditions could reach concentrations that would seriously interfere with the determination of protein in plasma. In spinal fluid, where the protein concentration is very low, it would appear more likely that there would be conditions in which the presence of drugs or other interfering substances might invalidate the method.

SUMMARY

An ultraviolet spectrophotometric method for the determination of proteins is described. It has advantages over other methods in simplicity, rapidity, accuracy, specificity, and sensitivity. Applications to the determination of total protein and albumin in plasma and to the determination of protein in cerebrospinal fluid and pleural fluid are described. The method is not useful for the determination of protein in urine.

I am indebted to Miss Gloria Nassif and Mrs. Jean Morgan for performance of the Kjeldahl determinations.

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COMPARISON OF THREE MEDIA FOR THE PRIMARY ISOLATION OF MYCOBACTERIUM TUBERCULOSIS

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INTRODUCTION

FOR the routine culture of *Mycobacterium tuberculosis*, the use of at least two media has been recommended. In order that an optimal procedure for the detection of tubercle bacilli in sputa and in gastric washings of patients might be established, an attempt was made to evaluate the results obtained by the use of three different media. The following media were used, the American Trudeau Society medium (ATS), the modified Löwenstein-Jensen medium (LJ), and a penicillin-blood agar medium (PBA). The use of blood agar medium was proposed by Tarshis and Frisch[1] in this country and independently by Reichert[2] in Germany.

MATERIALS AND METHODS

The methods of preparing the three types of media were as follows:

ATS Medium.—Potato flour,* 4.0 Gm.; 2.0 per cent glycerol in distilled water, 100 ml.; egg yolk, 100 ml.; and 2.0 per cent malachite green (50 per cent alcoholic solution), 2.0 ml. The potato flour and glycerol water were mixed, autoclaved at 15 pounds pressure for 15 minutes, and allowed to cool. The egg yolks were separated aseptically, homogenized, and added to the potato flour suspension. The ingredients were agitated and filtered through several layers of gauze. The dye was added approximately one hour before the medium was dispensed in sterile screw-cap test tubes. After inspissation, this medium was incubated for 48 hours at 37° C. to check for sterility.

LJ Medium.—Monopotassium phosphate, 2.4 Gm.; magnesium phosphate, 0.24 Gm.; magnesium citrate, 0.6 Gm.; asparagine (Difco), 3.6 Gm. glycerol, 12.0 ml.; distilled water, 600.0 ml.; potato flour, 30.0 Gm.; fresh whole eggs, 1,000.0 ml.; and 2.0 per cent malachite green (aqueous solution), 20.0 ml. The salts and aspargine were dissolved in the glycerol water, the potato flour was added, and the mixture was autoclaved at 15 pounds pressure for 15 minutes. The whole eggs were homogenized, added to the cooled potato flour–salt mixture, and filtered through several layers of gauze. The medium was treated in the same manner as ATS from this point.

PBA Medium.—This medium was prepared as suggested by Tarshis (personal communication). Blood agar base (Difco), 3.0 Gm.; glycerol (analytic grade), 1.0 ml.; outdated blood-bank blood (with citric acid solution), 30.0 ml.; distilled water, 69.0 ml.; penicillin (G potassium), 100 units per milliliter. The agar was dissolved in the glycerol water by heating and autoclaved at 15 pounds pressure for 15 minutes. After cooling

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to 56° C. in a water bath, the blood and penicillin were added aseptically and the ingredients mixed thoroughly. The medium was dispensed in sterile screw-cap test tubes in approximately 3.0 ml. amounts and allowed to solidify in a slanted position. Several tubes of medium were incubated for 48 hours at 37° C. to check for sterility.

All media were stored in the refrigerator until needed.

Specimens were sent to the laboratory in sterile, screw-cap bottles. Each specimen was digested in its entirety by the addition of an equal volume of sterile 4.0 per cent NaOH and agitation of it in a Kahn shaker for 10 minutes. After digestion, specimens were centrifuged, the supernatant decanted into a disinfectant, and the sediment titrated to neutrality with hydrochloric acid, with use of bromthymol blue as the indicator.

The media were arranged in two series, each consisting of one tube of ATS, LJ, and PBA, in order to determine whether or not the order of inoculation affects the number of positive isolations. Those inoculated first were designated the "A" series, the others inoculated last the "B" series. The sediment was thoroughly mixed before inoculating each tube to insure even distribution of the inoculum. The excess moisture was decanted from each tube and about 0.1 ml. of the sediment was inoculated on each slant. The tubes were incubated horizontally for 24 hours and then vertically for the remaining period of incubation. The cultures were examined weekly and those showing growth were removed for further examination. All cultures were held for eight weeks before being reported as negative. Smears were made of all positive cultures to check for acid-fast bacilli.

The data were analyzed statistically by the Chi square test.[3]

This study was conducted in a 300-bed tuberculosis hospital. The patients were either diagnosed as or suspected of having pulmonary tuberculosis. Those receiving chemotherapy were treated with varying combinations of the following antituberculous agents: streptomycin, para-aminosalicylic acid, isonicotinic acid hydrazide, viomycin, pyrazinamide, and terramycin.

RESULTS

A total of 1,782 specimens was planted on two tubes each of ATS, LJ, and PBA. Twenty-seven and three-tenths per cent of the total number of specimens supported growth on at least one of the six tubes inoculated and was, therefore, considered positive. The contamination rate was 4.0 per cent.

On 1,119 sputum specimens, smears of concentrated specimens were prepared at the same time cultures were inoculated. Out of the total specimens, 12.5 per cent was positive by smear, while 26.7 per cent was positive by culture. Positive smears were confirmed by positive cultures in 11.5 per cent of the cases, while 15.2 per cent of the positive cultures did not have positive smears. Only 1.0 per cent of positive smears was not confirmed by culture.

TABLE I. POSITIVE CULTURES AND CONTAMINATED SPECIMENS

	POSITIVES		CONTAMINANTS	
	NUMBER	PER CENT	NUMBER	PER CENT
Total specimens			1,782	100.0
All three media (duplicate tubes of each)	487	100.0	72	4.0
A Series (PBA, ATS, and LJ single tubes)	456	93.6	92	5.2
B Series (PBA, ATS, and LJ single tubes)	452	92.8	84	4.7
PBA + ATS (duplicate tubes of each)	476	97.7	84	4.7
PBA + LJ (duplicate tubes of each)	442	90.8	77	4.3
ATS + LJ (duplicate tubes of each)	462	94.9	116	6.5
PBA only (duplicate tubes)	400	82.1	120	6.7
ATS only (duplicate tubes)	409	84.0	225	12.6
LJ only (duplicate tubes)	371	76.2	169	9.5

A tabulation of the results on 487 positive cultures is given in Table I. Two series were inoculated in succession, the A and the B series, respectively, consisting of one tube each of ATS, LJ, and PBA. With use of 487 positive cultures as 100 per cent, the A series supported 93.6 per cent positive cultures and the B series 92.8 per cent. Inoculation of duplicate tubes of ATS and PBA gave 97.7 per cent positive cultures. Other combinations of media gave fewer positive cultures. The use of a single medium yielded a lower rate of positive cultures—84.0 per cent on ATS, 76.2 per cent on LJ, and 82.1 per cent on PBA—than the use of two or more media.

DISCUSSION

The usefulness of the blood agar medium as proposed by Tarshis and Frisch[1] has been confirmed by several investigators.[4-9] Hsiung and Haley[6] detected earlier growth on penicillin-blood agar (PBA) microscopically than on Löwenstein's (LJ), but macroscopic growth was observed at approximately the same time on both media. Our cultures were examined at weekly intervals, and growth, when present, appeared on all media at approximately the same time. However, larger colony formation appeared on PBA and, therefore, was easier to detect. Tarshis and associates[10] and Hosty and co-workers[5] found the growth of the tubercle bacilli on PBA to be characteristic for this organism and easily distinguishable from the growth of acid-fast saprophytes and chromogens. We observed the same characteristics of growth. Acid-fast saprophytes and chromogens either did not grow or were inhibited on PBA.

The percentage of positive and contaminated cultures was not significantly different in the two series of one tube of each medium. A few more positive cultures were obtained in the A series, which received inoculations first, than in the B series, but this difference is not statistically significant. Chi square = 0.05, $P < 0.9$. Therefore, the sequence in which the media were inoculated did not interfere with the number of positive isolations. The use of single tubes of three different media yielded fewer positive cultures than the use of duplicate tubes of ATS and PBA (Table I). However, this difference is not statistically significant.

The use of duplicate tubes of ATS and PBA did not yield significantly lower results than the use of duplicate tubes of three media. Chi square = 0.34, $P < 0.7$. The same is true for the combination ATS and LJ. Again the yield of this combination as compared with the use of three media is not statistically significant. Chi square = 1.77, $P < 0.2$. However, the use of the combination PBA and LJ yielded significantly lower results than the use of three media. Chi square = 5.68, $P < 0.02$, or the probability of this occurring by chance is less than 2 in 100.

If the medium producing the greatest percentage of positive cultures is compared with the optimal combination of two media, the advantage of using

two media becomes evident. The use of ATS alone yielded significantly lower results than the combination ATS and PBA. Chi square = 12.86, $P < 0.001$ or the probability of this occurring by chance is less than 1 in 1,000. Similarly, the use of PBA alone yielded significantly lower results than the combination PBA and ATS. Chi square = 16.56, $P < 0.001$.

If the three different media are compared when used singly on duplicate tubes, we find that the difference between ATS and PBA is not significant. Chi square = 0.26, $P < 0.70$. However, LJ compares unfavorably with ATS. The difference between the number of positive cultures yielded by these two media is significant. Chi square = 4.58, $P < 0.05$.

The contamination rate remained low when more than one type of medium was used. However, the contamination rate increased noticeably when a single medium was used. Contamination of PBA was less frequent than ATS and LJ when calculated individually (Table I). The difference in the rate between PBA and ATS (Chi square = 56.08) and between PBA and LJ (Chi square = 15.70) is highly significant; in both cases $P < 0.001$.

SUMMARY

The results obtained by the use of American Trudeau Society medium, modified Löwenstein-Jensen medium, and a penicillin-blood agar medium proposed by Tarshis, for the detection of *M. tuberculosis* on 1,782 specimens of sputum and gastric washings, are analyzed. The use of both penicillin-blood agar and American Trudeau Society media yields only 2.3 per cent less positive cultures than the use of all three media. The combination of these two media is, therefore, recommended for the routine detection of *M. tuberculosis*. If laboratory facilities permit the use of one medium only, penicillin-blood agar is recommended because of the ease of preparation, economy, ease of recognizing the growth of tubercle bacilli, high yield of positive cultures, and low rate of contamination.

We are grateful to Mrs. Betty Harbuck for her help in verifying and recording results, and to Miss Caroline Sullivan and Mr. Walter Irvin for their technical assistance.

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A MICROFORMALDEHYDOGENIC METHOD FOR THE ANALYSIS OF CHROMATOGRAPHIC FRACTIONS OF ADRENAL STEROIDS

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FUKUSHIMA and co-workers[6] have recently isolated from normal urine significant quantities of nonreducing adrenocortical steroid metabolites. Such compounds are not detected by the commonly employed reducing methods, or by the Porter-Silber reagent.[7] They are measurable by the periodate oxidation procedure introduced by Corcoran and Page.[2] This paper describes a simplified method which permits direct measurement of liberated formaldehyde and obviates the need for distillation or diffusion procedures. It has proved extremely useful in the analysis of large numbers of fractions collected during column chromatography.

EXPERIMENTAL

Reagents.—Potassium periodate,* 0.001 M in 0.3 N sulfuric acid; 23 mg. in 100.0 ml. of 0.3 N sulfuric acid.

Sodium bisulfite, Meta, Baker's analyzed, 10 per cent solution in water.

Glacial acetic acid, Merck reagent.

Chromotropic acid, Eastman sodium 1, 8-dihydroxynaphthalene-3, 6-disulfonate, recrystallized from 95 per cent ethanol. Stock solution contains 500.00 mg. per 4.0 ml. water and is stored at 4.0° C. This is diluted, 0.4 ml. stock solution to 10.0 ml. with concentrated sulfuric acid, Merck reagent, immediately before use.

Steroid standards, compounds E (Δ4-pregnene,11,20-trione-17α,21-diol), F (Δ4-pregnene-3,20-dione-11β,17α,21-triol), or B (Δ4-pregnene-3,20-dione-11β,21-diol) in free, unacetylated form may be employed and are generally available. These may be stored in suitable concentrations dissolved in ethanol and evaporated to dryness before use.

Procedure.—1. To the dry steroid residue, containing 2 to 30 mcg. of steroid, in a test tube 15 × 1.5 cm., is added 0.1 ml. glacial acetic acid. The tube is gently rolled to achieve solution of the steroid. Standards (3 to 20 mcg.) and appropriate blanks are carried through the same procedure.

2. To each tube is added 0.4 ml. 0.001 M periodate reagent, and oxidation is allowed to proceed for 45 minutes at room temperature.

3. To each tube is added exactly 0.1 ml. 10 per cent sodium bisulfite.

4. After the addition of 1.0 ml. chromotropic acid reagent, the tubes are placed in a boiling water bath for 30 minutes.

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5. The contents are then transferred to optically matched test tubes, 10 × 75 mm. and read in the Coleman Junior spectrophotometer at 560 mμ against the blank. Readings may also be made at 500 and 620 in order to assure maximal absorption at 560 attributable to formaldehyde and, if desired, may be corrected according to the principles of Allen.[1]

RESULTS AND DISCUSSION

The method described employs principles utilized previously but eliminates the necessity of distillation, as described by Daughaday and associates[3] and Corcoran and Page,[2] or diffusion, according to Wilson.[8] The latter techniques are impractical for the analysis of large numbers of chromatographic fractions.

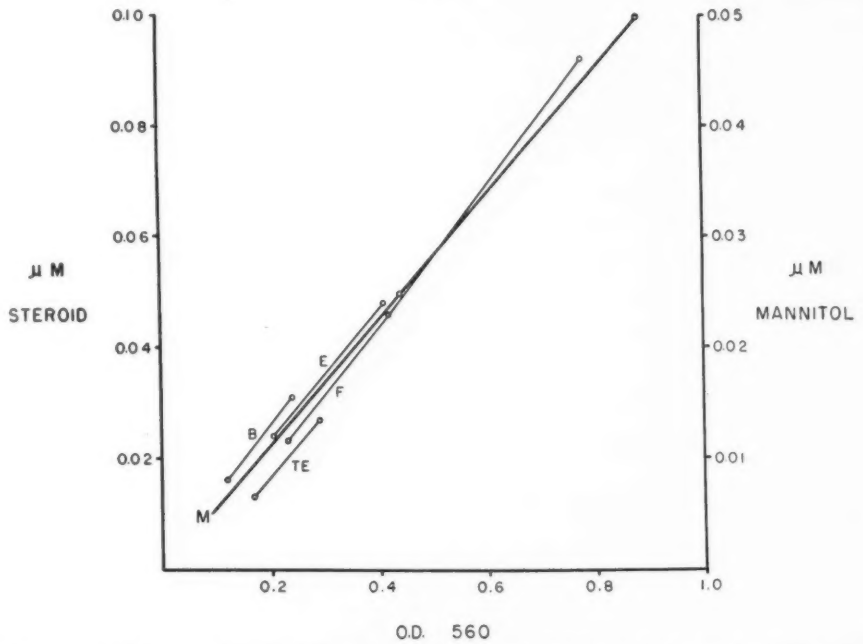

Fig. 1.—Micromoles of various steroids (compounds E,B,F, and tetrahydro-E) are plotted on the left ordinate, mannitol (M) on the right ordinate, and the optical density along the abscissa. The scale for mannitol is adjusted to ½ that of the steroids since each mole of the former yields two moles of formaldehyde.

In order that these steps might be eliminated it was necessary to use bisulfite as the reductant for the excess periodate, since it was shown by Frissell and associates[5] that optically clear solutions are obtained in this fashion. The bisulfite also binds the formaldehyde produced. In addition, a minimal concentration of periodate, as recommended, avoids the production of interfering chromogens upon the addition of chromotropic and sulfuric acid. Theoretically, this amount of periodate is sufficient to oxidize approximately 350 mcg. of steroids with an alpha-ketolic side-chain or 175 mcg. of steroids with 17,20,21-triol groups. The final concentration of sulfuric acid achieved, namely, approximately 9 M, is critical for the maximal development of color with chromotropic acid. The addition of thiourea, as suggested by Frissell,[5] has not offered any advantages with the described technique.

Fig. 1 illustrates the reliability of the method, showing the employment of several pure corticoids and comparison of the yields of formaldehyde with those of mannitol. It is to be noted that each mole of mannitol yields two moles of formaldehyde, whereas only one mole of formaldehyde is produced by the oxidation of one mole of appropriate steroid. In all instances, the colors produced exhibited maximal absorption at 560 mμ, typical of the formaldehyde complex. It was also found that the employment of a correction factor according to Allen,[1] by readings at 500, 560, and 620 mμ, yields results which obey Beer's law (Fig. 2), although in this instance there is not close agreement with the values obtained with mannitol. Nonetheless, with the employment of steroid standards, the relationship is good, and such a correction may be applied when the color development does not appear to be entirely satisfactory. However, this simplified method is not recommended for the estimation of formaldehydogenic steroids in residues from crude extracts of biologic fluids.

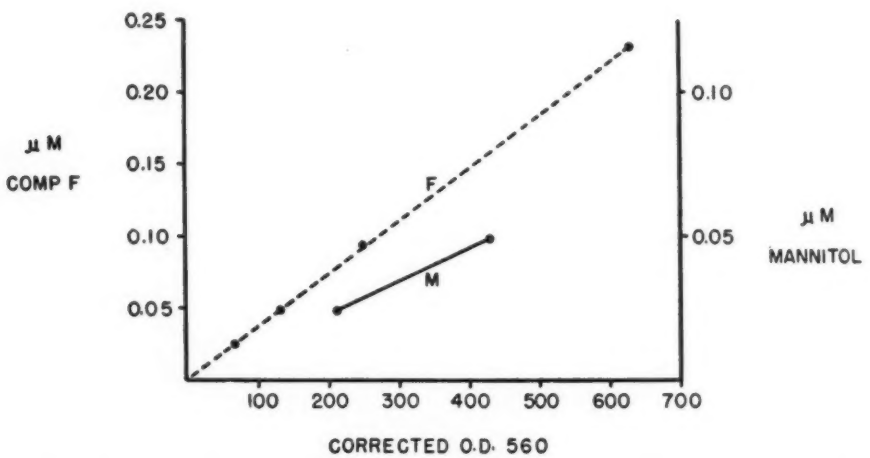

Fig. 2.—Various concentrations of compound F (plotted as micromoles along the ordinate) are indicated as corrected optical densities at 560 mμ along the abscissa (corrected reading = reading at 560 − $\frac{500 + 620}{2}$). mannitol (M).

The method has permitted us[4] to estimate the quantities of steroids present in fractions collected during the chromatography of adrenocortical steroids from human urine and on one occasion has led to the detection of a new compound, pregnane-3α,17α,20(?),21-tetrol, which would have escaped notice had only reduction methods been employed.

SUMMARY

A simplified method for the determination of formaldehydogenic steroids, applicable to the analysis of numerous chromatographic fractions, has been described.

We acknowledge with gratitude the technical and artistic assistance of Mr. Frank Rosalia.

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THE COLLECTION OF URINE FOR AMMONIA DETERMINATION

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IN STUDIES of the ammonia excretion of guinea pigs and rabbits, animals which excrete an alkaline urine, it was found impossible to obtain reliable 24-hour collections.[1] A review of the literature revealed that others had encountered similar difficulties,[2] and the impression was gained that the paucity of information on the excretion of ammonia in alkaline urines might chiefly be due to this obstacle. The problems encountered were adequate separation of urine from feces and prevention of enzymatic splitting of urea.

For the separation of urine from feces, several methods have been published, from the simple mesh screen[3] to an elaborate stocklike cage.[4] In most studies the use of a mesh screen was satisfactory and simple and could be used for the study of large numbers of animals. The commonly used methods for the separation of urine from feces have recently been reviewed by Lazarow.[5]

The second problem, contamination with and growth of urease-forming organisms, had not previously been satisfactorily solved. In 1860, Pasteur[6] demonstrated that urine was frequently contaminated with urea-splitting organisms. As demonstrated by Musculus,[7] in 1874, "ammoniacal fermentation" due to urease from decomposed microorganisms also occurred in urine freed of bacteria by filtration. To prevent growth, boric acid or combinations of thymol or phenyl mercuric nitrate with mineral oil or toluene have been used by others, but we have not been able to get uncontaminated urine using this method. To evaluate the usefulness of various methods, a number of experiments were performed, and a new technique was devised which permitted urine collections over longer periods of time without decomposition of the urea.

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EXPERIMENTAL

Guinea pigs, which normally have urine of pH 7.5 to 8.5, were used. For collection of 24-hour urines they were placed in commercial rat metabolism cages of the type depicted in figure 2 of the article by Lazarow.[5] A screen and glass-wool filter were used for the separation of feces from urine. For collection of catheter specimens, only females were used, and the urine was collected under oil to diminish loss of carbon dioxide. Ammonia determinations were done by the method of Seligson.[8] The pH was determined with a Cambridge Model R pH meter, and no temperature correction was employed.

RESULTS AND DISCUSSION

Six animals were catheterized and placed in metabolic cages, and the urine was collected for 24 hours under mineral oil containing thymol, after which they were catheterized again. Ammonia was determined and the values for the 24-hour specimens were compared with those of the catheter specimens. In order that comparable ammonia excretion values might be obtained, the urine flow was assumed to be constant throughout each 24-hour period. Mean ammonia excretion during the 24-hour collection period was 1.40 μeq per minute with a range of 0.070 to 4.70, while the specimen obtained previously by catheter showed a mean of 0.010 and a range of 0.001 to 0.029 μeq per minute and that obtained afterward a mean of 0.025 with a range of 0.001 to 0.061 μeq per minute. The pH of the 24-hour specimens varied from 8.3 to 8.8, but the before-and-after collections were in the 7.5 to 8.3 range. It is evident that wider variations and higher pH and ammonia values were observed in the 24-hour collections than in specimens obtained by catheterization.

To exclude the possibility of a marked day-night variation and to get information on the "true" ammonia output, six animals were catheterized every 3 hours for a period of 24 hours. After the first 3 collection periods micturition occurred between catheterizations so that urine flows and ammonia output per minute were not reliable. After trying various methods to obtain 24-hour urine collections in the rabbit, White and Rolf[2] also resorted to catheterization, but this method proved unreliable as well as cumbersome. The first few collection periods of our experiment probably afforded a fairly accurate estimate of ammonia output which was corroborated by later acute experiments. The pH of these samples varied from 6.95 to 8.15 and the ammonia excretion from 0.001 to 0.011 μeq per minute with a mean of 0.003.

Urine was collected by catheter from a number of anesthetized guinea pigs. For the present purpose, only the first three collection periods were used, since these probably give the best picture of ammonia excretion. The values ranged from 0.008 to 0.085 μeq per minute with a mean of 0.025.

There was close agreement of the mean rates of ammonia excretion in the urines obtained by catheterization in the above experiments; therefore, these

results seemed to be acceptable as an estimate of the rate of ammonia excretion. It was felt, however, that none of these methods was satisfactory for 24-hour collections.

Musculus[9] showed that hydrochloric and other acids readily inactivated the nonorganized ferment which increased the ammonia and decreased the urea content of urine allowed to stand. The following experiment was carried out.

Experiment.—The urine from each cage was collected in a jar containing 20 ml. of 1 N hydrochloric acid. The clear urine-acid mixture at the end of 24 hours showed pH 1.0, and no bacterial growth was evidenced. To obtain information on the titratable acidity or alkalinity of the urine, the mixture was titrated electrometrically to pH 7.4 with sodium hydroxide and corrected for the added acid and volume. The ammonia output of 6 animals over two consecutive 24-hour collection periods was determined by this method, and the results are shown in Table I.

TABLE I. THE RATE OF EXCRETION OF AMMONIA BY GUINEA PIGS, DETERMINED FROM CAGE URINE COLLECTED IN ACID

EXP. NO.	1ST 24 HR. COLLECTION		2ND 24 HR. COLLECTION	
	URINE FLOW ML./MIN.	AMMONIA EXCRETION μEQ/MIN.	URINE FLOW ML./MIN.	AMMONIA EXCRETION μEQ/MIN.
1	0.024	0.011	0.017	0.042
2	0.010	0.021	0.008	0.030
3	0.012	0.008	0.021	0.031
4	0.010	0.008	0.010	0.021
5	0.009	0.018	0.004	0.026
6	0.017	0.015	0.011	0.037
Mean	0.01380	0.01343	0.01193	0.03088

The small individual variation of ammonia output is conspicuous. It should be mentioned that the increase on the second day was probably due to the feeding of cabbage. Not only is there little individual variation with this method, but the data obtained agree closely with the results from catheter specimens. An obvious objection to this method was that it was impossible to determine the urine pH; however, titratable acidity and ammonia gave adequate information for our purposes.

Since the pK of ammonia is 9.4 (Conway[10]), urines at pH 8, 8.5, and 9.0 will contain 3.8, 11.2, and 28.5 per cent of their ammonia in unionized form. It seemed necessary, therefore, to determine the ammonia lost while the urine is running down the cage funnel.

Two milliliter aliquots of analyzed, fresh, pooled guinea pig urine were run down a metabolism cage which had been occupied by an animal for several hours. The samples were collected and analyzed. This experiment showed that 5 per cent of the volume was lost though this value would tend to decrease with increasing micturition volume. The loss of ammonia was approximately 10 per cent at urine pH over 8.0. It decreased with increasing ionization of ammonia and was zero at pH 6.3. There is no convenient way to avoid this ammonia loss.

and it should be kept in mind that, with this method, the actual ammonia of alkaline urines would be greater than the amount found. This was the opposite of what had been observed with the conventional collection methods. In spite of the small losses it was felt that the method was better than those previously used.

To compare the various methods of urine preservation the following experiment was done.

Experiment.—Urine samples collected under oil by catheterization of different animals were analyzed immediately for ammonia and were divided into three portions which were placed in 1 N HCl, thymol-mineral oil or an open container with no preservative. At the end of 48 hours all were analyzed for ammonia. The results are presented in Table II.

TABLE II. THE EFFECT OF STANDING ON THE AMMONIA CONTENT OF GUINEA PIG URINE WITHOUT PRESERVATIVE COMPARED WITH THYMOL-MINERAL OIL AND HYDROCHLORIC ACID

TIME OF STANDING	PRESERVATIVE	μM NH_3/ML. URINE				
0 hours	None	0.44	0.68	1.26	1.26	1.26
48 hours	1 N HCl	0.29	1.59	1.26	1.26	1.26
48 hours	Mineral oil + thymol	6.17	0.59	12.6	11.6	13.6
48 hours	None	7.94	0.59	9.5	9.5	9.2

This experiment demonstrates both the variability and the lack of reliability of conventional methods of collecting urine samples for ammonia analysis and also the superiority of collecting in acid. The concentration of ammonia in samples with no preservative or with thymol-mineral oil was usually but not necessarily increased but was not changed on standing in acid.

SUMMARY

Since alkaline urines are frequently contaminated with ammonia-producing organisms, the conventional urine collection methods are not reliable for determination of 24-hour ammonia excretion. To overcome this obstacle, a method was devised whereby the collection was made in acid. The method proved to be convenient and reliable.

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